

01-12-00

A

**UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)***(Only for new nonprovisional applications under 37 CFR 1.53(b))*Docket No.
989.6351DIVTotal Pages in this Submission
61**TO THE ASSISTANT COMMISSIONER FOR PATENTS**Box Patent Application
Washington, D.C. 20231

Transmitted herewith for filing under 35 U.S.C. 111(a) and 37 C.F.R. 1.53(b) is a new utility patent application for an invention entitled:

Family of Mammalian Potassium Channels, Their Cloning and Their Use, Especially For The Screening of Drugs

and invented by:

Florian Lesage, et al.

If a **CONTINUATION APPLICATION**, check appropriate box and supply the requisite information:☐ Continuation ☒ Divisional ☐ Continuation-in-part (CIP) of prior application No.: 08/749,816

Which is a:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No.:

Which is a:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No.:

Enclosed are:

Application Elements

1. ☒ Filing fee as calculated and transmitted as described below
2. ☒ Specification having 28 pages and including the following:
 - a. ☒ Descriptive Title of the Invention
 - b. ☐ Cross References to Related Applications (if applicable)
 - c. ☐ Statement Regarding Federally-sponsored Research/Development (if applicable)
 - d. ☐ Reference to Microfiche Appendix (if applicable)
 - e. ☒ Background of the Invention
 - f. ☒ Brief Summary of the Invention
 - g. ☒ Brief Description of the Drawings (if drawings filed)
 - h. ☒ Detailed Description
 - i. ☒ Claim(s) as Classified Below
 - j. ☒ Abstract of the Disclosure

UTILITY PATENT APPLICATION TRANSMITTAL (Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.
989.6351DIV

Total Pages in this Submission
61

Application Elements (Continued)

3. ☒ Drawing(s) (when necessary as prescribed by 35 USC 113)
- a. ☐ Formal b. ☒ Informal Number of Sheets 11
4. ☒ Oath or Declaration
- a. ☐ Newly executed (original or copy) ☐ Unexecuted
- b. ☒ Copy from a prior application (37 CFR 1.63(d)) (for continuation/divisional application only)
- c. ☐ With Power of Attorney ☐ Without Power of Attorney
- d. ☐ DELETION OF INVENTOR(S)
Signed statement attached deleting inventor(s) named in the prior application,
see 37 C.F.R. 1.63(d)(2) and 1.33(b).
5. ☒ Incorporation By Reference (usable if Box 4b is checked)
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied
under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby
incorporated by reference therein.
6. ☐ Computer Program in Microfiche
7. ☒ Genetic Sequence Submission (if applicable, all must be included)
- a. ☐ Paper Copy
- b. ☐ Computer Readable Copy
- c. ☒ Statement Verifying Identical Paper and Computer Readable Copy

Accompanying Application Parts

8. ☐ Assignment Papers (cover sheet & documents)
9. ☐ 37 CFR 3.73(b) Statement (when there is an assignee)
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure Statement/PTO-1449 ☐ Copies of IDS Citations
12. ☒ Preliminary Amendment
13. ☒ Acknowledgment postcard
14. ☒ Certificate of Mailing
- ☐ First Class ☒ Express Mail (Specify Label No.): EL525816853US

UTILITY PATENT APPLICATION TRANSMITTAL (Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.
989.6351DIV

Total Pages in this Submission
61

Accompanying Application Parts (Continued)

15. ☐ Certified Copy of Priority Document(s) (if foreign priority is claimed)
16. ☐ Small Entity Statement(s) - Specify Number of Statements Submitted: _____
17. ☒ Additional Enclosures (please identify below):

Associate Power of Attorney
Request to Use Computer Readable Form

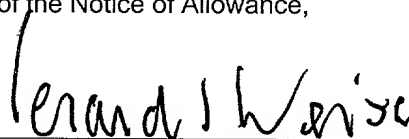
Fee Calculation and Transmittal

CLAIMS AS FILED

For	#Filed	#Allowed	#Extra	Rate	Fee
Total Claims	12	- 20 =	0	x \$9.00	\$0.00
Indep. Claims	1	- 3 =	0	x \$39.00	\$0.00
Multiple Dependent Claims (check if applicable) <input type="checkbox"/>					\$0.00
BASIC FEE					\$345.00
OTHER FEE (specify purpose) _____					\$0.00
TOTAL FILING FEE					\$345.00

- ☒ A check in the amount of **\$345.00** to cover the filing fee is enclosed.
- ☒ The Commissioner is hereby authorized to charge and credit Deposit Account No. **13-3405** as described below. A duplicate copy of this sheet is enclosed.
- ☐ Charge the amount of _____ as filing fee.
- ☒ Credit any overpayment.
- ☒ Charge any additional filing fees required under 37 C.F.R. 1.16 and 1.17.
- ☐ Charge the issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance, pursuant to 37 C.F.R. 1.311(b).

Dated: January 11, 2000


Signature

Gerard J. Weiser
Reg. No. 19,763
Schnader Harrison Segal & Lewis LLP
1600 Market Street - Suite 3600
Philadelphia, PA 19103-7286

CC:

CERTIFICATE OF MAILING BY "EXPRESS MAIL" (37 CFR 1.10)Applicant(s): **Florian Lesage, et al.**

Docket No.

989.6351DIV

Serial No.

Not yet known

Filing Date

January 11, 2000

Examiner

Not yet Assigned

Group Art Unit

Not yet Assigned

Invention: **Family of Mammalian Potassium Channels, Their Cloning and Their Use, Especially For The Screening of Drugs**

I hereby certify that this **Divisional Application and accompanying documents**

(Identify type of correspondence)

is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 in an envelope addressed to: The Assistant Commissioner for Patents, Washington, D.C. 20231

on **January 11, 2000**

(Date)

Kim Sheehan

(Typed or Printed Name of Person Mailing Correspondence)

Kim Sheehan

(Signature of Person Mailing Correspondence)

EL525816853US

("Express Mail" Mailing Label Number)

Note: Each paper must have its own certificate of mailing.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Art Unit : 1600 Market Street
Examiner : Suite 3600
Serial No. : Not yet known Philadelphia, PA 19103
Filed : January 11, 2000
Inventor : Florian Lesage, et al. Docket: 989.6351DIV

Title : Family of Mammalian Potassium Channels, Their Cloning And
: Their Use, Especially For The Screening of Drugs

Dated: January 11, 2000

PRELIMINARY AMENDMENT

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination , please amend the application as follows:

IN THE SPECIFICATION:

Please incorporate the attached Sequence Listing into the specification of the present application.
This Sequence Listing is identical with that filed in parent application Serial No. 08/749,816, filed
November 15, 1996.

IN THE CLAIMS:

Delete claims 49-53 and 58-67.

Add the following claims:

--68. A method of screening for a substance capable of modulating the activity of a mammalian protein comprising 2 P domains and 4 transmembrane segments, which protein is competent to transport potassium across a membrane, comprising contacting pre-selected amounts of the substance to be tested with cells expressing the potassium transport channel, measuring the effects of said substance on the potassium transport activity of the protein, and identifying the substance that has a positive or negative effect on said transport activity.

69. The method of claim 68 wherein the protein which is competent to transport potassium across a membrane is human.

70. The method of claim 68 wherein the cell expressing the potassium transport protein is transformed with a self replicating vector comprising a nucleic acid sequence encoding a mammalian protein comprising 2 P domains and 4 transmembrane segments, which protein is competent to transport potassium across a membrane.

71. The method of claim 70 wherein the self replicating vector comprises a nucleic acid sequence encoding a human potassium transport protein.

72. The method of claim 71 wherein the self replicating vector comprises SEQ ID. No. 1.

73. A substance, identified by the method of claim 68, which is capable of positively or negatively influencing the transport activity of a potassium transport channel.

74. The substance of claim 73 which influences the transport activity of the potassium transport channel comprising 2 P domains and 4 transmembrane segments.

75. The substance of claim 74 which influences the transport activity of the potassium transport channel represented by SEQ ID. No. 2.

76. A pharmaceutical composition for the treatment of diseases caused by the malfunction of a potassium transport channel, comprising the substance of claim 73.

77. The pharmaceutical composition of claim 76 which influences the transport activity of a potassium transport channel comprising 2 P domains and 4 transmembrane segments.

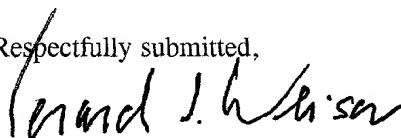
78. The pharmaceutical composition of claim 77 which influences the transport activity of the potassium transport channel represented by SEQ ID. No. 2.

79. The pharmaceutical composition of claim 76 which is useful for the treatment of diseases selected from the group consisting of epilepsy, heart arrhythmias, vascular diseases, neurodegenerative diseases, ischemia or anoxia, endocrine diseases associated with anomalies of hormone secretion, and muscle diseases. -

REMARKS

This Preliminary Amendment is being filed to add claims directed to a screening method using the potassium channels "twik" and cells expressing such channels.

Respectfully submitted,



Gerard J. Weiser
Reg. No. 19,763
SCHNADER HARRISON SEGAL & LEWIS LLP
Attorneys for Applicants

NEW FAMILY OF MAMMALIAN POTASSIUM CHANNELS, THEIR CLONING AND THEIR USE, ESPECIALLY FOR THE SCREENING OF DRUGS

The present invention relates to a new family of potassium channels. More specifically, the invention relates to the cloning of a human potassium channel that constitutes the first member of a new functional and structural group of potassium channels. The abundance of this channel and its presence in a large number of tissues are such as to confer on it a fundamental role in the transport of potassium in a large number of types of cells.

Potassium channels are ubiquitous in eukaryote and prokaryote cells. Their exceptional functional diversity make them ideal candidates for a large number of biological processes in living cells (Rudy, B., 1988, *Neurosciences*, 25, 729-749; Hille, B., 1992, *"Ionic Channels of Excitable Membrane"*, 2nd edition, Sinauer, Sunderland, Massachusetts). In excitable cells, the K^+ channels define the form of the action potentials and the frequency of the electric activity, and play a major role in neuronal integration, muscle contraction or hormonal secretion. In nonexcitable cells, their expression appears to be correlated with specific stages of the development of the cell (Barres, B. A. et al., 1990, *Annu. Rev. Neurosci.*, 13, 441-474). In most cells, specific types of K^+ channels play a vital role in determining the electrical potential of the membrane at rest by regulating the membrane permeability to K^+ ions. These channels exhibit the characteristic of being instantaneous and open in a large range of membrane potentials.

Recent cloning studies have resulted in the identification of a large number of subunits capable of forming potassium channels (Betz, H., 1990, *Biochemistry*, 29, 3591-3599; Pongs, O., 1992, *Physiol. Rev.*, 72, S69-88; Salkoff, L. et

al., 1992, *Trends Neurosci.*, 15, 161-166; Jan, L. Y. and Y. N. Jan, 1994, *Nature*, 371, 199-122; Doupnik, C. A. et al., 1995, *Curr. Opin. Neurobiol.*, 5, 268-277) which could be regulated by other types of subunits (Aldrich, R. W., 1994, *Curr. Biol.*, 4, 839-840; Isom, L. L. et al., 1994, *Neuron*, 12, 1183-1194; Rettig, J. et al., 1994, *Nature*, 369, 289-294; Attali, B. et al., 1995, *Proc. Natl. Acad. Sci. USA*, 92, 6092-6096).

The subunits of the voltage-dependent K⁺ channels activated by depolarization (Kv families) and the calcium-dependent K⁺ channels exhibit six hydrophobic transmembranal domains, one of which (S4) contains repeated positive charges which confer on these channels their sensitivity to voltage and, consequently, in their functional outward rectification (Logothetis, D. E. et al., 1992, *Neuron*, 8, 531-540; Bezanilla, F. and Stefani, E., 1994, *Annu. Rev. Biophys. Biomol. Struct.*, 23, 819-846).

The K⁺ channels with inward rectification (Kir families) have only two transmembranal domains. They do not have the S4 segment and the inward rectification results from a voltage-dependent blockade by cytoplasmic magnesium (Matsuda, H., 1991, *Annu. Rev. Physiol.*, 53, 289-298; Lu, Z. and Mackinnon, R., 1994, *Nature*, 371, 243-246; Nichols, C. G. et al., 1994, *J. Physiol. London*, 476, 399-409).

A common structural unit, called the P domain, is found in both groups, and constitutes an essential element of the structure of the K⁺-permeable pore. The presence of this unit in a membrane protein is considered to be the signature of the structure of a K⁺ channel (Pongs, O., 1993, *J. Membrane Biol.*, 136, 1-8; Heginbotham, L. et al., 1994, *Biophys. J.*, 66, 1061-1067; Mackinnon, R., 1995, *Neuron*, 14, 889-892; Pascual, J. M. et al., 1995, *Neuron*, 14, 1055-1063).

The present invention is based on the cloning of a K⁺ channel which is the first member of a new structural and functional group of potassium channels.

This new K⁺ channel has a novel molecular architecture with four transmembranal segments and two P domains. From a functional point of view, this channel is remarkable in that it exhibits weak inward rectification properties. This new channel is referred to below as TWIK-1 following the English-language term "Tandem of P domains in a Weak Inward rectifying K⁺ channel". Its abundance and its presence in a large number of tissues are such as to confer on it a fundamental role in the transport of potassium in a large number of types of cells.

The discovery of this new family of potassium channels and the cloning of a member of this family provides, notably, new means for screening drugs capable of modulating the activity of these new potassium channels and thus of preventing or treating the diseases in which these channels are involved.

The research activities that led to the cloning of the TWIK-1 channel were carried out in the manner described below with reference to the attached sequences and drawings in which:

- SEQ ID NO: 1 represents the nucleotide sequence of the cDNA of TWIK-1 and the amino acid sequences of the coding sequence.

- SEQ ID NO: 2 represents the amino acid sequence of the TWIK-1 protein.

- Figure 1 represents the Northern blot analysis, the nucleotide sequences and the deduced amino acid sequence, as well as the hydrophobicity profile of TWIK-1. (a): expression of TWIK-1 mRNA in human tissues; each track contains 5 µg of poly(A)⁺; the autoradiograph was exposed for 24 hours. (b) cDNA sequence of TWIK-1 and the amino acid sequences of the coding sequence. The supposed transmembranal segments are circled and the P domains are underlined; o represents a potential glycosylation site and ■ represents the threonine residue in the consensus recognition site of protein kinase C. (c): the hydrophobicity analysis and the topology of TWIK-1 deduced from it; the

hydrophobicity values were calculated according to the method of Kyte and Doolittle (window size of 11 amino acids) and are presented in relation to the position of the amino acid; the shaded hydrophobic peaks correspond to the transmembranal segments.

- Figure 2 represents the sequence alignments. (a): alignment of the P domains of TWIK-1, TOC/YORK and other representative K⁺ channel families; the identical and conserved residues are circled in black and in gray, respectively. (b): alignment of TWIK-1 with potential homologues of *C. elegans*; the sequences M110.2 and F17C8.5 were deduced from the gene sequences (respective access numbers Z49968 and Z35719); the computerized splicing of the other genomic sequences of *C. elegans* (respective access numbers Z49889, P34411 and Z22180) is not sufficiently precise to allow their perfect alignment and is therefore not shown.

- Figure 3 shows the biophysical and pharmacological properties of K⁺ currents recorded by the imposed voltage technique on *Xenopus* oocytes that had received an injection of TWIK-1 cRNA; (a): the oocyte was maintained at a holding potential (HP) of -80 mV and the currents were recorded at the end of 1-s voltage jumps from -120 to +60 mV in 20 mV increments. (b): regular current-voltage relationship using the same technique as in (a). (c): potential reversal of the TWIK-1 currents (E_{rev}) as a function of the external K⁺ concentration. (d) current tracings linked to +30 mV depolarizations starting at a holding potential (HP) of -80 mV in the absence (top tracing) and in the presence (bottom tracing) of 1 mM of Ba²⁺. (e): blocking effect of 100 μ M of quinine, same protocol as in (d). (f) dose-response relationship of the blocking of the TWIK-1 currents by quinine.

- Figure 4 shows the influence of the expression of TWIK-1 on the membrane potential. (a): dose-response relationships of the cRNA; top row =

equilibrium state of the outward currents measured at +30 mV; bottom row = membrane potentials associated with the resting state. (b): effect of 100 μ M of quinine on the membrane potential of an oocyte which did not receive an injection (left tracing) and that of an oocyte that received 20 ng of TWIK-1 cRNA. (c): statistical evaluation of the depolarizing effects of 100 μ M of quinine on oocytes that did not receive injections (left bars) and on oocytes that received injections of 20 ng of TWIK-1 cRNA (right bars); control (unfilled bar), + quinine (solid bars); each bar represents the mean \pm SD of 5 oocytes.

- Figure 5 shows the properties of the single TWIK-1 channel. (a): current tracings recording in the input-output configuration to the membrane potentials indicated in the absence (m) or in the presence (·) of internal M^{2+} (3 mM) and in symmetry with 140 mM of K^+ . (b): mean of curves I-V (n = 10). (c and d): open time of distribution obtained at +80 mV (top histograms) and at -80 mV (bottom histograms) in the presence of 3 mM Mg^{2+} (c) or in the absence of Mg^{2+} (d).

- Figure 6 shows the blocking of the TWIK-1 channels by the internal pH. (a and b): blocking effect of the internal acidification on the TWIK-1 currents, induced by perfusion of CO_2 ; (a) tracings of superimposed currents induced by a depolarization phase at -30 mV starting at HP = -80 mV, control (top tracing), effect when equilibrium is reached in the presence of CO_2 (bottom tracing); (b): graph (n = 5) showing the almost complete blockade of the TWIK-1 currents induced by CO_2 ; (c and d): internal acidification induced by the application of DNP (1 mM). (c): same protocol as in (a), control (top tracing) and after 5 minutes of application of DNP (bottom tracing); (d): graph (n = 4) indicating the percentage of TWIK-1 current remaining after treatment with DNP. (e and f): imposed voltage (method: attached patch) under symmetrical conditions of K^+ concentration (140 mM) maintained at +80 mV. (e) course over

time of the effect of 1 mM of DNP (marked with arrow) on the activities of the single TWIK-1 channel. (f): graph ($n = 4$) showing the effect of DNP on the mean probability of opening NP_o calculated during 1 minute of recording starting at the equilibrium state. (g): activities measured in the "inside-out-patch" state at 80 mV at different internal pH values. Bar graph ($n = 10$) of NP_o in relation to the internal pH.

- Figure 7 shows the activation of the TWIK-1 channels by PMA, activator of protein kinase C. (a): perfusion of PMA (30 nM) for 10 minutes increases the TWIK-1 current (top tracing) induced by a depolarization phase at +30 mV starting at HP = -80 mV, control current (top tracing). (b): graph ($n = 5$) showing the activation effect of PMA on the TWIK-1 currents. (c and d): attached patch configuration under symmetrical K^+ concentration conditions maintained at +60 mV; (c): course over time of the effect of 30 nM of PMA on the single channel activities; the recordings of the channel activity were performed with a rapid scanning before and after the application of PMA; (d): bar graph ($n = 5$) showing the activation effect of PMA on NP_o .

The P domains of K^+ channels were used to determine the corresponding sequences in the GenBank data base by means of the BLAST sequence alignment program (Altschul, S. F. et al., 1990, *J. Mol. Biol.*, 215, 403-410). There was thus identified a 298 pb human Tag expressed sequence (EST, HSC3AH031), the deduced amino acid sequence of which includes a nonconventional "P-like" domain sequence: GLG in place of GYG as shown in figure 2a. It was then envisaged that this EST sequence was a partial copy of a mRNA coding a new type of K^+ channel subunit. A DNA probe was prepared from this sequence in order to carry out hybridization with a Northern blot (Clontech) of multiple human tissues. A 1.9 kb transcript was thereby found in abundance, as shown in figure 1a, in the heart and the brain and, at lower

levels, in the placenta, the lung, the liver and the kidney. The DNA probe was used to screen a bank of kidney cDNA and four independent clones were obtained. The cDNA inserts of 1.8 to 1.9 kb of these clones all have the same open reading frame (ORF) containing a regio identical to the 298 pb sequence of HSC3AH031 and differing solely in the length of their noncoding 5' sequences.

Primary Structure of TWIK-1

The following characteristics were demonstrated:

- The sequences of the cDNA clones contain an ORF of 1011 nucleotides coding for a polypeptide of 336 amino acids shown in figure 1b.

- This protein has two P domains.

- Other than the P domains, no significant alignment was seen between TWIK-1 and a K⁺ channel recently cloned in yeast and which also has two P domains (Ketchum, K. A. et al., 1995, *Nature*, 376, 690-695).

- Analysis of the hydrophobicity of TWIK-1, shown in figure 1c, reveals the presence of four transmembranal domains, designated T1 to T4.

- By placing the NH2 end on the cytoplasmic surface, in accordance with the absence of signal peptide, one obtains the topology model shown in figure 1c.

- In this model, the two P domains are inserted in the membrane from the exterior in accordance with the known orientation of these loops in the K⁺ channels.

- In addition, the general structural unit of TWIK-1 is similar to the unit that one would obtain by making a tandem of two classical subunits rectifying the entry of a potassium channel. Like a classical inward rectifier, TWIK-1 does not exhibit the highly conserved segment S4 which is responsible for the

sensitivity to the membrane potential of the inward rectification of the K⁺ channels of the Kv family.

- A nonusual large loop of 59 amino acids is present between M1 and P1, such as to extend the length of the linker M1-P1 of the extracellular side of the membrane.

- A potential site of N-glycosylation is present in this loop.

- Three consensus sites of phosphorylation are present at the N-terminal (Ser 19 for calcium calmodulin kinase II) and C-terminal (Ser 303 for casein kinase II) ends of the cytoplasmic domains, and in the M2-M3 linker (Thr161 for protein kinase II).

- The alignment of the P domains of an important group of K⁺ channels is presented in figure 2a. It shows that the regions constituting the pore selective for K⁺ are well conserved including the G residues in position 16 and 18 and three other residues indicating practically exclusively conservative changes in positions 7, 14 and 17. It is of interest to note that a leucine residue is present in the place of a tyrosine conserved in position 18 in the P2 domain of TWIK-1, or of a phenylalanine in position 17 of the P domain of the K⁺ channel of type eag.

The homologues of TWIK-1

Comparison of the complete sequence of TWIK-1 with the sequences of the Genbank data base allowed identification of at least five genes of *Caenorhabditis elegans* which had been characterized in the context of the Nematode Sequencing project, and which potentially code for structural homologues of TWIK-1. The alignment of two of these homologues with TWIK-1 is shown in figure 2b. The homologies of total sequences between the deduced proteins of *C. elegans* and TWIK-1 are circa 55 to 60% and circa 25

to 28% of identity. The homologies among sequences of *C. elegans* are not higher.

Functional expression of TWIK-1

For the functional study, the coding sequence of TWIK-1 was inserted between the noncoding sequences 5' and 3' of *Xenopus* globin in the vector pEXO (Lingueglia, E. et al., 1993, *J. Biol. Chem.*, 269, 13736-13739). A complementary RNA (cRNA) was transcribed of this construction and injected in the oocytes of *X. laevis*. A noninactivating current, free from noninjected cells, was measured by the imposed voltage technique, as shown in figure 3a. Kinetic activation of the current is usually instantaneous and cannot be resolved because it is masked by the capacitive discharge of the current recorded at the beginning of the impulse. The current-voltage relationship is linear above 0 mV and then saturates for a stronger depolarization of the membrane, as shown in figure 3b. TWIK-1 is therefore K⁺ selective. In the case of a replacement of the external K⁺ by Na⁺ or N-methyl-D-glucuronate, the reversal of the potential of the currents follows the K⁺ equilibrium potential (E_K), as shown in figure 3c. In addition, a change by 10 in the concentration [(K)]_o leads to a change of 56 ± 2 mV in the inversion value of the potential, in accordance with Nernst's equation.

As shown in figure 3, the K⁺ currents of TWIK-1 are inhibited by Ba²⁺ (figure 3d) with an IC₅₀ value of 100 μM, by quinine (figure 3e and 3f) and by quinidine (not shown) with respective IC₅₀ values of 50 and 95 μM. The TWIK-1 currents are slightly sensitive to TEA and to the class III antiarrhythmic agent tedisamil (30% inhibition for each, at 20 mM and 100 μM, respectively). Less than 10% inhibition was seen after application of 4-aminopyridine (1 mM), apamin (0.3 μM), charybdotoxine (3 nM), dedrotoxine (0.1 μM),

clofilium (30 μ M), amiodarone (100 μ M) and glibenclamide (30 μ M). The TWIK-1 channel is not sensitive to the K⁺ channel openers cromakaline (100 μ M) and pinacidil (100 μ M).

Figure 4 shows the effect of increasing the doses of injected TWIK-1 cRNA on the independent expression of the time of the K⁺ currents and on the resting state of the membrane potential (E_m). As soon as the current appears, the oocytes become increasingly polarized, reaching a value of E_m close to E_K . The amplitude of the TWIK-1 current reaches values of 0.6 to 0.8 μ M with the injection of 20 ng per oocyte. Higher doses of TWIK-1 cRNA are toxic, leading to a reduction in expression. In oocytes that received 20 ng of cRNA, quinine is the best blocker of TWIK-1, inducing a noteworthy reversible depolarization (73 ± 6 mV, $n = 5$) as shown in figures 4b and 4c.

The unitary properties of the TWIK-1 channel

Single channel current recordings, shown in figure 5, in an inside-out patch configuration or in a whole cell configuration show that the TWIK-1 channels assure the passage of influx or exit currents as a function, respectively, of a depolarization or a hyperpolarization (figure 5a). The current-voltage relationship of the single channel, shown in figure 5 b, shows a barely accentuated inward rectification in the presence of 3 mM (figure 5) and 10 mM (not shown) of Mg^{2+} on the cytoplasmic side. As shown in figure 5b, this rectification disappears in the absence of internal M^{2+} . With 3 mM of internal Mg^{2+} , the mean duration of opening at +80 mV is 1.9 ms and the unitary conductance is 19 ± 1 pS (figure 5c). At -80 mV, the channels are oscillating with a mean duration of opening of 0.3 ms, and a conductance value increasing to $34 \pm$ pS. The withdrawal of the internal Mg^{2+} ions does not influence the kinetic parameters under either polarized or depolarized conditions,

but the unitary conductance measured at -80 mV reaches 35 ± 4 pS. This apparent increase in conductance in the single channel suggests that it is the extremely rapid oscillation induced by Mg^{2+} that results in an underestimation of the real value of conductance. The same properties were observed in the fixed cell configuration, showing that the channel behavior is not modified by the excision of the patch. The TWIK-1 channels in the excised patches do not discharge and do not appear to be deficient in intracellular constituents. In contrast to numerous channels which require the presence of ATP for their activity in the excised patch configuration, ATP is not required for the expression of TWIK-1. In addition, perfusion of the patch with a solution containing 10 mM of ATP does not induce any effect on the activity of the TWIK-1 channel.

The activity regulation properties of the TWIK-1 channel.

The intracellular pH (P_{Hi}) is involved in the control of numerous cellular processes, and in cells such as the hepatic cells, the change in P_{Hi} regulates the membrane potential (Bear, C. E. et al., 1988, *Biochim. Biophys. Acta*, 944, 113-120).

Intracellular acidification of the oocytes was produced using two methods:

- superfusion with a solution enriched in CO_2 which produces acidification by a mechanism involving the bicarbonate transport system (Guillemare, E. et al., 1995, *Mol. Pharmacol.*, 47, 588-594);
- treatment with dinitrophenol (DNP), which is a metabolic inhibitor that decouples the H^+ gradient in mitochondria and induces internal acidity (Pedersen, P. L. and Carafoli, E., 1987, *Trends Biol. Sci.*, 12, 146-189).

Both of these experimental methods resulted in a significant reduction in the TWIK-1 currents, greater than 95% in the case of CO_2 and 80% in the case

of DNP of the control amplitude values, as shown in figures 6a to 6d. The inhibition induced by DNP on the activity of the single K^+ channel was again observed under the attached patch conditions, as shown in figures 6e to 6f. However, after excision of the patch, the activity of the channel became insensitive to the acidification of the internal solution produced either by modifying the Na_2HPO_4/NaH_2PO_4 buffer ratio (figures 6g and 6h) or by bubbling of CO_2 (not shown). Thus, the effect of the pH value on the activity of the TWIK-1 channel is probably indirect.

Phosphorylation or dephosphorylation of specific amino acid residues is an important mechanism of regulation of the ionic channels (Levitan, I. B., 1994, *Annu. Rev. Physiol.*, 56, 193-212). As shown in figure 7, activation of protein kinase C by phorbol-12 myristate acetate (PMA, 30 nM) increases the TWIK-1 currents. The inactive phorbol ester 4α -phorbol-12, 13 didecanoate (PDA, 1 μ M) has no effect. In an attached patch which initially expressed solely a single channel, application of PMA ... the presence of at least five channels (figure 7c and 7d). This experiment shows that at least four channels are silently present in the patch before the application of PMA. Since the TWIK-1 sequence contains a consensus phosphorylation site for protein kinase C (PKC), located at the level of the threonine in position 161 (figure 1b), the effect of PMA suggests regulation under the control of PKC. However, the mutation of the threonine 161 into alanine leads to a muted channel which remains functional and conserves the capacity to be activated by PMA.

Activation of protein kinase A by application of 8-Cl-AMPc (300 μ M) or forskolin (10 μ M) does not affect the activity of TWIK-1. Elevation of the cytoplasmic Ca^{2+} concentration by application of A23187 (1 μ M) which could be activated by Ca^{2+} -calmodulin kinase II and/or reveal the presence of a channel

activated by the Ca^{2+} , is also without effect on the properties of the TWIK-1 channel.

Thus, the object of the present invention is an isolated, purified nucleic acid molecule that codes for a protein constituting a TWIK-1 potassium channel or exhibiting the properties and structure of the type of the TWIK-1 channel described above.

More specifically, the said nucleic acid molecule codes for the TWIK-1 protein, the amino acid sequence of which is represented in the attached sequence list as number SEQ ID NO: 2, or a functionally equivalent derivative of this sequence. Such derivatives can be obtained by modifying and or suppressing one or more amino acid residues of this sequence, as long as this modification and/or suppression does not modify the functional properties of the TWIK-1 potassium channel of the resultant protein.

The sequence of a DNA molecule coding for this protein is more specifically the molecule coding for TWIK-1 represented in the attached sequence list as number SEQ ID NO: 1.

The invention also relates to a vector containing a molecule of the aforementioned nucleic acid, as well as a procedure for production or expression in a cellular host of a protein constituting a TWIK-1 potassium channel or a channel of the same family as TWIK-1.

A procedure for production of a protein constituting a TWIK-1 potassium channel or exhibiting the properties and structure of the type of the TWIK-1 channel consists of:

- transferring a nucleic acid molecule of the invention or a vector containing the said molecule into a cellular host,

- culturing the cellular host obtained in the preceding step under conditions enabling the production of potassium channels exhibiting the properties of TWIK-1,

- isolating by any suitable method the proteins constituting the potassium channels of the TWIK-1 family.

A procedure for expression of a TWIK-1 potassium channel or a potassium channel of the same family as TWIK-1 consist of:

- transferring a nucleic acid molecule of the invention or a vector containing the said molecule into a cellular host,

- culturing the cellular host obtained in the preceding step under conditions enabling the expression of potassium channels of the TWIK-1 family.

The cellular host employed in the preceding procedures can be selected from among the prokaryotes or the eukaryotes, and notably from among the bacteria, the yeasts, mammal cells, plant cells or insect cells.

The vector used is selected in relation to the host into which it will be transferred; it can be any vector such as a plasmid.

The invention thus also relates to the transferred cells expressing the potassium channels exhibiting the properties and structure of the type of the TWIK-1 channel obtained in accordance with the preceding procedures.

The cells expressing TWIK-1 potassium channels or channels exhibiting the properties and structure of the type of the TWIK-1 channels obtained in accordance with the preceding procedures are useful for the screening of substances capable of modulating the activity of the TWIK-1 potassium channels. This screening is carried out by bringing into contact variable amounts of a substance to be tested with cells expressing the TWIK-1 channel or potassium channels exhibiting the properties and structure of the type of the TWIK-1

channels, then measuring, by any suitable means, the possible effects of said substance on the currents of the potassium channels of these channels.

This screening procedure makes it possible to identify drugs that useful in the treatment of diseases of the heart or of the nervous system. Diseases involving the potassium channels and thus likely to involve the channels of the TWIK-1 family are, for example, epilepsy, heart (arrhythmias) and vascular diseases, neurodegenerative diseases, especially those associated with ischemia or anoxia, the endocrine diseases associated with anomalies of hormone secretion, muscle diseases.

An isolated, purified nucleic acid molecule coding for a protein constituting a TWIK-1 potassium channel or a vector including this nucleic acid molecule or a cell expressing the TWIK-1 potassium channels, are also useful for the preparation of transgenic animals. These can be animals supra-expressing the said channels, but especially so-called knock-out animals, i.e., animals presenting a deficiency of these channels; these transgenic animals are prepared by methods known to the experts in the field, and enable the preparation of live models for studying animal diseases associated with the TWIK-1 channels.

The nucleic acid molecules of the invention or the cells transformed by said molecule can also be used in genetic therapy strategies for compensating for a deficiency in the potassium channels at the level of one or more tissues of a patient. The invention thus also relates to a medication containing nucleic acid molecules of the invention or cells transformed by said molecule for the treatment of disease involving the potassium channels.

In addition, the gene of the TWIK-1 channel has been located on chromosome 1 at position q42-q43. The chromosomal localization of this gene constitutes a determinant result for the identification of genetic diseases associated

with this new family of potassium channels; thus, the knowledge of the structure of the TWIK-1 family of channels is such as to allow performance of a prenatal diagnosis of such diseases.

The present invention also has as its object a new family of K⁺ channels, of which TWIK-1 is a member, which are present in most human tissues and especially abundant in the brain and the heart, and which exhibit the properties and structure of the type of those of the TWIK-1 channels described above. Thus it relates to an isolated, purified protein whose amino acid sequence is represented in the attached sequence list as number SEQ ID NO: 2, or a functionally equivalent derivative of this sequence.

Such derivatives can be obtained by modifying and/or suppressing one or more amino acid residues of this sequence or by segmenting this sequence, as long as this modification and/or suppression or deletion of a fragment does not modify the functional properties of the TWIK-1 type potassium channel of the resultant protein.

A protein constituting a TWIK-1 type potassium channel is useful for the manufacture of medications intended for the treatment or prevention of diseases involving dysfunction of the potassium channels.

Polyclonal or monoclonal antibodies directed against a protein constituting a TWIK-1 type potassium channel can be prepared by the conventional methods described in the literature.

These antibodies are useful for investigating the presence of potassium channels of the TWIK-1 family in different human or animal tissues, but they can also find applications in the therapeutic domain, due to their specificity, for the *in vivo* inhibition or activation of TWIK-1 type potassium channels.

Other advantages and characteristics of the invention will be made obvious from the examples below which are nonlimitative examples related to the cloning and expression of TWIK-1.

Identification of the HSC3AH031 EST sequence and analysis of the RNA

The P domains of the cloned channels were used to investigate homologues in the NCBI (National Center of Biotechnology) data bases using the sequence alignment program tBLASTn. Translation of an EST sequence (HSC3AH031, Genbank access number: F12504) presented a significant sequence similarity ($P = 1.2 \times 10^{-3}$) with the second P domain of a yeast K^+ channel. This 298 pb sequence was originally obtained from a human brain cDNA bank in the context of the Genexpress cDNA program (Auffray, C. et al., 1995, *C. R. Acad. Sci., III, Sci. Vie*, 318, 263-272). A 255 pb DNA fragment corresponding to HSC3AH031 was amplified by PCR from cDNA derived from human brain poly(A)⁺ and subcloned in pBluescript (Stratagene) to yield pBS-HSC3A.

For the RNA analysis, a Northern blot of multiple human tissues (Clontech) was screened with the pBS-HSCA insert tagged with P^{32} in 50% formamide, 5 x SSPE (0.9 M NaCl; 50 mM sodium phosphate; pH 7.4; 5 mM EDTA), 0.1% SDS, 5 x Denhardts, 20 mM potassium phosphate, pH 6.5 and 250 µg of salmon sperm DNA denatured at 55°C for 18 hours. The blots were washed to a final stringency of 0.1 SSC (3 M NaCl; 0.3 M sodium citrate; pH 7.0), 0.3% SDS at 65°C.

Isolation of the cDNA cloning TWIK-1

An oligo(dT) cDNA bank stemming from poly(A)⁺ RNA isolated from human adult kidney was screened with the pBS-HSCA insert tagged with P^{32} .

00491990-04400

The filters were hybridized in 50% formamide, 5 x SSC, 4 x Denhardt, 0.1% SDS and 100 µg of salmon sperm DNA denatured at 50°C for 18 hours. Four positive hybridization clones were isolated from circa 5 x 10⁵ clones. The λZAPII phages containing the cDNA inserts were converted into cDNA plasmids (Stratagene). The DNA inserts were characterized by restriction enzyme analysis and by total or partial DNA sequencing on both strands using the dideoxy nucleotide method on an automated sequencer (Applied Biosystems 373A).

Mutations, cRNA synthesis and oocyte injection.

The TWIK-1 coding sequence was amplified using a low-error rate DNA polymerase (Pwo DNA pol, Boehringer) and subcloned in the plasmid pEXO so as to yield pEXO-TWIK-1. Mutations were performed using the whole plasmid pEXO-TWIK-1 with a highly reliable PCR extension kit (Boehringer) and two adjacent primers. One of these introduced a punctiform mutation in the TWIK-1 coding sequence, changing the 161 Thr codon into a codon for alanine. The product of the PCR was linearized by the enzyme BamHI and the cRNA were synthesized using a T7 RNA polymerase (Stratagene). Preparation of the *X. laevis* oocytes and cRNA injection were carried out in accordance with the literature (Guillemare, E. et al., 1992, *Biochemistry*, 31, 12463-12468).

Electrophysiological measurements.

In a 0.3-ml perfusion chamber, a single oocyte was impaled on two standard glass microelectrodes (0.5 - 2.0 MW) charged with 3 M KCl and maintained under voltage-clamp with a Dagan TEV200 amplifier. The bath solution contained 98 mM KCl, 1.8 mM CaCl₂, 2 mM MgCl₂ and 5 mM HEPES at

pH 7.4 with KOH. Stimulation of the preparation, data acquisition and analyses were carried out with the pClamp program (Axon Instruments, USA).

For the patch-clamp experiments, the vitelline membrane was removed from the oocytes as described in the literature (Duprat, F. et al., 1995, *Biochem. Biophys. Res. Commun.*, 212, 657-663); the oocytes were then placed in a bath solution containing 140 mM KCl, 1.8 mM CaCl_2 , 2 mM MgCl_2 and 5 mM HEPES at pH 7.4 with KOH. The pipettes were filled with a strong K^+ solution (40 mM KCl, 100 mM of potassium methane sulfonate, 1.8 mM CaCl_2 , 2 mM MgCl_2 and 5 mM HEPES adjusted to pH 7.4 with KOH). 100 μM of GdCl_3 was added to the pipette solution to inhibit the action of the activated channels. The inside-out patches were perfused with a solution containing 140 mM KCl, 10 mM CaCl_2 , 5 mM HEPES adjusted to pH 7.2 with KOH and 5 mM EGTA added daily. The single channel signals were filtered at 3.5 kHz and analyzed with the Biopatch program (Bio-Logic, Grenoble, France).

LIST OF SEQUENCES.

[Key: See Pages 21-24]

INFORMATION REGARDING SEQ ID NO: 1

I - CHARACTERISTIC OF THE SEQUENCE:

A) LENGTH:

B) TYPE

C) STRAND NUMBER:

D) CONFIGURATION:

II - TYPE OF MOLECULE:

XI - SEQUENCE DESCRIPTION: SEQ ID NO: 1

INFORMATION REGARDING SEQ ID NO: 2

I - CHARACTERISTIC OF THE SEQUENCE:

A) LENGTH:

B) TYPE

C) STRAND NUMBER:

D) CONFIGURATION:

II - TYPE OF MOLECULE:

XI - SEQUENCE DESCRIPTION: SEQ ID NO: 21

LISTE DE SÉQUENCES.

INFORMATION CONCERNANT LA SEQ ID NO:1 :

I - CARACTERISTIQUE DE LA SEQUENCE :

- A) LONGUEUR :
- B) TYPE :
- C) NOMBRE DE BRIN :
- D) CONFIGURATION :

II - TYPE DE MOLECULE :

XI - DESCRIPTION DE SEQUENCE : SEQ ID NO:1 :

GGGCAGGAAG ACGGCGCTGC CCGGAGGAGC GGGGCGGGCG GGCGCGCGGG GGAGCGGGCG	60
GCGGGCGGGA GCCAGGCCCG GGCGGGGGCG GGGGCGGCGG GGCCAGAAGA GGCGGCGGGC	120
CGCGCTCCGG CCGGTCTGCG GCGTTGGCCT TGGCTTTGGC TTTGGCGGCG GCGGTGGAGA	180
AG ATG CTG CAG TCC CTG GCC GGC AGC TCG TGC GTG CGC CTG GTG GAG CGG	230
Met Leu Gln Ser Leu Ala Gly Ser Ser Cys Val Arg Leu Val Glu Arg	
1 5 10 15	
CAC CGC TCG GCC TGG TGC TTC GGC TTC CTG GTG CTG GGC TAC TTG CTC	278
His Arg Ser Ala Trp Cys Phe Gly Phe Leu Val Leu Gly Tyr Leu Leu	
20 25 30	
TAC CTG GTC TTC GGC GCA GTG GTC TTC TCC TCG GTG GAG CTG CCC TAT	326
Tyr Leu Val Phe Gly Ala Val Val Phe Ser Ser Val Glu Leu Pro Tyr	
35 40 45	
GAG GAC CTG CTG CGC CAG GAG CTG CGC AAG CTG AAG CGA CGC TTC TTG	374
Glu Asp Leu Leu Arg Gln Glu Leu Arg Lys Leu Lys Arg Arg Phe Leu	
50 55 60	
GAG GAG CAC GAG TGC CTG TCT GAG CAG CAG CTG GAG CAG TTC CTG GGC	422
Glu Glu His Glu Cys Leu Ser Glu Gln Gln Leu Glu Gln Phe Leu Gly	
65 70 75 80	
CGG GTG CTG GAG GCC AGC AAC TAC GGC GTG TCG GTG CTC AGC AAC GCC	470
Arg Val Leu Glu Ala Ser Asn Tyr Gly Val Ser Val Leu Ser Asn Ala	
85 90 95	
TCG GGC AAC TGG AAC TGG GAC TTC ACC TCC GCG CTC TTC TTC GCC AGC	518
Ser Gly Asn Trp Asn Trp Asp Phe Thr Ser Ala Leu Phe Phe Ala Ser	
100 105 110	
ACC GTG CTC TCC ACC ACA GGT TAT GGC CAC ACC GTG CCC TTG TCA GAT	566
Thr Val Leu Ser Thr Thr Gly Tyr Gly His Thr Val Pro Leu Ser Asp	
115 120 125	
GGA GGT AAG GCC TTC TGC ATC ATC TAC TCC GTC ATT GGC ATT CCC TTC	614
Gly Gly Lys Ala Phe Cys Ile Ile Tyr Ser Val Ile Gly Ile Pro Phe	
130 135 140	

- 22 -

CATATGTGAC AAAATTATCT CGACCTTACA TAGGAGGAGA ATACTTGAAG CAGTATGCTG 1483
 CTGTGGTTAG AAGCAGATTT TATACTTTTA ACTGGAAACT TTGGGGTTTG CATTAGATC 1543
 ATTTAGCTGA TGGCTAAATA GCAAAATTTA TATTTAGAAG CAAAAAAAAA AAGCATAGAG 1603
 ATGTGTTTTA TAAATAGGTT TATGTGTACT GGTTCATG TACCCACCCA AAATGATTAT 1663
 TTTTGGAGAA TCTAAGTCAA ACTCACTATT TATAATGCAT AGGTAACCAT TAACTATGTA 1723
 CATATAAAGT ATAAATATGT TTATATTCTG TACATATGGT TTAGGTCACC AGATCCTAGT 1783
 GTAGTTCTGA AACTAAGACT ATAGATATTT TGTTCCTTTT GATTTCTCTT TATACTAAAG 1843
 AATCCAGAGT TGCTACAATA AAATAAGGGG AATAATAAAA AAAAAAAAAA A 1894

INFORMATION CONCERNANT LA SEQ ID NO :2

I - CARACTERISTIQUE DE LA SEQUENCE :

- A) LONGUEUR :
- B) TYPE :
- C) NOMBRE DE BRIN :
- D) CONFIGURATION :

II - TYPE DE MOLECULE :

XI - DESCRIPTION DE LA SEQUENCE : SEQ ID NO:2 :

Met Leu Gln Ser Leu Ala Gly Ser Ser Cys Val Arg Leu Val Glu Arg
 1 5 10 15
 His Arg Ser Ala Trp Cys Phe Gly Phe Leu Val Leu Gly Tyr Leu Leu
 20 25 30
 Tyr Leu Val Phe Gly Ala Val Val Phe Ser Ser Val Glu Leu Pro Tyr
 35 40 45
 Glu Asp Leu Leu Arg Gln Glu Leu Arg Lys Leu Lys Arg Arg Phe Leu
 50 55 60
 Glu Glu His Glu Cys Leu Ser Glu Gln Gln Leu Glu Gln Phe Leu Gly
 65 70 75 80
 Arg Val Leu Glu Ala Ser Asn Tyr Gly Val Ser Val Leu Ser Asn Ala
 85 90 95
 Ser Gly Asn Trp Asn Trp Asp Phe Thr Ser Ala Leu Phe Phe Ala Ser
 100 105 110
 Thr Val Leu Ser Thr Thr Gly Tyr Gly His Thr Val Pro Leu Ser Asp
 115 120 125
 Gly Gly Lys Ala Phe Cys Ile Ile Tyr Ser Val Ile Gly Ile Pro Phe
 130 135 140
 Thr Leu Leu Phe Leu Thr Ala Val Val Gln Arg Ile Thr Val His Val
 145 150 155 160

- 24 -

CLAIMS

1) Isolated and purified nucleic acid molecule coding for a protein constituting a protein channel exhibiting the properties and structure of the TWIK-1 type channel.

2) Isolated and purified nucleic acid molecule coding for a protein constituting a potassium channel, characterized in that it codes for the protein the amino acid sequence of which is represented in the attached sequence list as number SEQ ID NO: 2 or a functionally equivalent derivative of this sequence.

3) Nucleic acid molecule according to claim 2, the sequence of which is represented in the attached sequence list as number SEQ ID NO: 1.

4) Vector containing a nucleic acid molecule according to one of claims 1 to 3.

5) Procedure for the production of a protein constituting a potassium channel exhibiting the properties and structure of the TWIK-1 type channel consisting of:

- transferring a nucleic acid molecule according to one of claims 1 to 3 or a vector according to claim 4, into a cellular host,

- culturing the cellular host obtained in the preceding step under conditions allowing the production of potassium channels exhibiting the properties of TWIK-1,

- isolating, by any suitable means, the proteins constituting the potassium channels exhibiting the properties and structure of the TWIK-1 type channel.

6) Procedure for the expression of a potassium channel exhibiting the properties and structure of the TWIK-1 type channel consisting of:

- transferring a nucleic acid molecule according to one of claims 1 to 3 or a vector according to claim 4, into a cellular host,
- culturing the cellular host obtained in the preceding step under conditions allowing the expression of potassium channels exhibiting the properties and structure of the TWIK-1 type channel.

7) Procedure according to one of claims 5 or 6, characterized in that the cellular host is selected from among the prokaryotes or the eukaryotes and, particularly, from among the bacteria, the yeasts, mammal cells, plant cells or insect cells.

8) Cell expressing the potassium channels exhibiting the properties and structure of the TWIK-1 type channel obtained by the procedure according to claim 6 or 7.

9) Procedure for screening substances capable of modulating the activity of the potassium channels of the TWIK-1 type channel, characterized in that:

one brings into contact variable amounts of a substance to be tested with the cells expressing the potassium channels exhibiting the properties and structure of the TWIK-1 type channel according to claim 8, then

- one measures, by any suitable means, the possible effects of said substance on the currents of the potassium channels exhibiting the properties and structure of the TWIK-1 type channel.

(10) Pharmaceutical composition for the compensation of a deficiency in the potassium channels at the level of one or more tissues, characterized in

that it comprises nucleic acid molecules according to one of claims 1 to 3, or a vector according to claim 4, or cells according to claim 8.

11) Isolated and purified protein constituting a potassium channel exhibiting the properties and structure of the TWIK-1 type channel.

12) Protein according to claim 11, the amino acid sequence of which is represented in the attached sequence list as number SEQ ID NO: 2, or a functionally equivalent derivative of this sequence.

13) Pharmaceutical composition for the compensation of a deficiency in the potassium channels at the level of one or more tissues, characterized in that it comprises a protein according to claim 11 or 12.

14) Monoclonal or polyclonal antibody directed against a protein according to claim 11 or 12.

NEW FAMILY OF MAMMALIAN POTASSIUM CHANNELS, THEIR CLONING AND THEIR USE, ESPECIALLY FOR THE SCREENING OF DRUGS

The present invention relates to the cloning of a member of a new potassium channel named TWIK-1. More specifically, it relates to an isolated and purified nucleic acid molecule coding for a protein constituting a potassium channel exhibiting the properties and structure of the TWIK-1 type channel, as well as the protein coded by this nucleic acid molecule.

The invention also relates to the use of this nucleic acid molecule to transform cells, and the use of these cells expressing the potassium channels exhibiting the properties and structure of the TWIK-1 type channel for the screening of drugs.

Heart Brain Placenta Lung Liver Sk. Muscle Kidney

FIG. 1A

1	1000	1000
2	1000	1000
3	1000	1000
4	1000	1000
5	1000	1000
6	1000	1000
7	1000	1000
8	1000	1000
9	1000	1000
10	1000	1000
11	1000	1000
12	1000	1000
13	1000	1000
14	1000	1000
15	1000	1000
16	1000	1000
17	1000	1000
18	1000	1000
19	1000	1000
20	1000	1000
21	1000	1000
22	1000	1000
23	1000	1000
24	1000	1000
25	1000	1000
26	1000	1000
27	1000	1000
28	1000	1000
29	1000	1000
30	1000	1000
31	1000	1000
32	1000	1000
33	1000	1000
34	1000	1000
35	1000	1000
36	1000	1000
37	1000	1000
38	1000	1000
39	1000	1000
40	1000	1000
41	1000	1000
42	1000	1000
43	1000	1000
44	1000	1000
45	1000	1000
46	1000	1000
47	1000	1000
48	1000	1000
49	1000	1000
50	1000	1000
51	1000	1000
52	1000	1000
53	1000	1000
54	1000	1000
55	1000	1000
56	1000	1000
57	1000	1000
58	1000	1000
59	1000	1000
60	1000	1000
61	1000	1000
62	1000	1000
63	1000	1000
64	1000	1000
65	1000	1000
66	1000	1000
67	1000	1000
68	1000	1000
69	1000	1000
70	1000	1000
71	1000	1000
72	1000	1000
73	1000	1000
74	1000	1000
75	1000	1000
76	1000	1000
77	1000	1000
78	1000	1000
79	1000	1000
80	1000	1000
81	1000	1000
82	1000	1000
83	1000	1000
84	1000	1000
85	1000	1000
86	1000	1000
87	1000	1000
88	1000	1000
89	1000	1000
90	1000	1000
91	1000	1000
92	1000	1000
93	1000	1000
94	1000	1000
95	1000	1000
96	1000	1000
97	1000	1000
98	1000	1000
99	1000	1000
100	1000	1000

GCC	TGG	TGC	TTC	GGC	TTC	CTG	GTG	CTG	GGC	TAC	TTG	CTC	TAC	CTG	GTC	TTC	GGC	GCA	114
A	W	C	F	G	F	L	V	L	G	Y	L	L	Y	L	V	F	G	A	38

GTG GTC TTC TCC TCG GTG GAG CTG CCC TAT GAG GAC CTG CTG CGC CAG GAG CTG CGC 171
V V F S S V E L P Y E D L L R Q E L R 57

AAG CTG AAG CGA CGC TTC TTG GAG GAG CAC GAG TGC CTG TCT GAG CAG CAG CTG GAG 228
K L K R R F L E E H E C L S E Q Q L E 76

CAG TTC CTG GGC CGG GTG CTG GAG GCC AGC AAC TAC GGC GTG TCG GTG CTC AGC AAC 285
Q F L G R V L E A S N Y G V S V L S N 95

GCC TCG GGC AAC TGG AAC TGG GAC TTC ACC TCC GCG CTC TTC TTC GCC AGC ACC GTG 342
A S G N W N W D F T S A L F F A S T V 114

CTC	TCC	ACC	ACA	GGT	TAT	GGC	CAC	ACC	GTG	CCC	TTG	TCA	GAT	GGA	GGT	AAG	GCC	TTC	399
L	S	T	T	G	Y	G	H	T	V	P	L	S	D	G	G	K	A	F	133

TGC	ATC	ATC	TAC	TCC	GTC	ATT	GGC	ATT	CCC	TTC	ACC	CTC	CTG	TTC	CTG	ACG	GCT	GTG	456
C	I	I	Y	S	V	I	G	I	P	F	T	L	L	F	L	T	A	V	152

GTC	CAG	CGC	ATC	ACC	GTG	CAC	GTC	ACC	CGC	AGG	CCG	GTC	CTC	TAC	TTC	CAC	ATC	CGC	513
V	Q	R	I	T	V	H	V	T	R	R	P	V	L	Y	F	H	I	R	171

TGG	GGC	TTC	TCC	AAG	CAG	GTG	GTG	GCC	ATC	GTC	CAT	GCC	GTG	CTC	CTT	GGG	TTT	GTC	570
W	G	F	S	K	Q	V	V	A	I	V	H	A	V	L	L	G	F	V	190

ACT	GTG	TCC	TGC	TTC	TTC	TTC	ATC	CCG	GCC	GCT	GTC	TTC	TCA	GTC	CTG	GAG	GAT	GAC	627
T	V	S	C	F	F	F	I	P	A	A	V	F	S	V	L	E	D	D	209

FIG. 1B

TGG AAC TTC CTG GAA TCC TTT TAT TTT TGT TTT ATT TCC CTG AGC ACC ATT GGC CTG	684
W N F L E S F Y F C F I S L S T I G L	228
GGG GAT TAT GTG CCT GGG GAA GGC TAC AAT CAA AAA TTC AGA GAG CTC TAT AAG ATT	741
G D Y V P G E G Y N Q K F R E L Y K I	247
GGG ATC ACG TGT TAC CTG CTA CTT GGC CTT ATT GCC ATG TTG GTA GTT CTG GAA ACC	798
G I T C Y L L L G L I A M L V V L E T	266
TTC TGT GAA CTC CAT GAG CTG AAA AAA TTC AGA AAA ATG TTC TAT GTG AAG AAG GAC	855
F C E L H E L K K F R K M F Y V K K D	285
AAG GAC GAG GAT CAG GTG CAC ATC ATA GAG CAT GAC CAA CTG TCC TTC TCC TCG ATC	912
K D E D Q V H I I E H D Q L S F S S I	304
ACA GAC CAG GCA GCT GGC ATG AAA GAG GAC CAG AAG CAA AAT GAG CCT TTT GTG GCC	969
T D Q A A G M K E D Q K Q N E P F V A	323
ACC CAG TCA TCT GCC TGC GTG GAT GGC CCT GCA AAC CAT TGA gcgtaggatttgttcatt	1030
T Q S S A C V D G P A N H *	337
atgctagagcaccagggtcaggggtcaaggaagaggcctaagtatgttcatttttatcagaatgcaaaagcgaaaa	1106
ttatgtcactttaagaaatagctactgtttgcaatgtcttattaaaaaacaacaaaaaagacacatggaacaaag	1182
aagctgtgaccccagcaggatgtctaatatgtgaggaatgagatgtccacctaaaattcatatgtgacaaaatta	1258
tctcgaccttacataggaggagaataacttgaagcagtatgctgtgtggttagaagcagattttatacttttaact	1334
ggaaactttggggtttgcatttagatcatttagctgatggcctaataagcaaaatttatatttgaagcaaaaaaaa	1410
aaagcatagagatgtgtttataaataggtttatgtgtactggtttgcatgtaccaccccaaatgattattttg	1486
gagaatctaagtcaaactcactatttataatgcataaggaaccattaactatgtacatatataaagtataaatatgtt	1562
tatatctgtacatatgtgttaggtcaccagatcctagtgtagtcttgaaactaagactatagatatatttgtttct	1638
tttgatttctctttataactaaagaatccagagttgtctacaataaaataaggggaataataaaaaaaaaaaaaa	1712

FIG. 1B

FIG. 1C

	1	14	27																								
TWIK-1 P1	E	T	S	A	L	E	F	A	S	T	V	L	S	T	I	G	Y	G	H	T	V	P	L	S	D	G	G
TWIK-1 P2	E	L	E	S	F	Y	F	C	F	I	S	L	S	T	I	G	L	G	D	Y	V	P	G	E	G	Y	N
TOK1 P2	Y	F	N	C	I	Y	F	C	F	L	C	L	L	T	I	G	Y	G	D	Y	A	P	R	T	G	A	G
TOK1 P1	Y	G	N	A	L	Y	F	C	T	V	S	L	L	T	V	G	L	G	D	I	L	P	K	S	V	G	A
Slo	Y	W	T	C	V	Y	E	L	I	V	T	M	S	T	V	G	Y	G	D	V	Y	C	E	T	V	L	G
Shaker	I	P	D	A	F	W	A	V	T	M	T	T	V	G	Y	G	D	M	T	E	V	G	F	W	G		
Shab	I	P	E	A	F	W	A	G	I	T	M	T	T	V	G	Y	G	D	I	C	P	T	A	L	G		
Shal	I	P	A	A	F	W	T	I	V	T	M	T	T	V	G	Y	G	D	M	V	P	E	T	A	G		
Shaw	I	P	L	G	L	W	A	L	V	T	M	T	T	V	G	Y	G	D	M	A	P	K	T	A	G		
KAT1	Y	V	T	A	L	Y	W	S	I	T	L	L	T	T	S	Y	G	D	F	H	A	E	N	P	R	E	
AKT1	Y	V	T	S	M	Y	W	S	I	T	L	L	T	T	V	G	Y	G	D	I	H	P	V	N	T	K	E
eag	Y	V	T	A	L	Y	F	T	M	T	C	M	T	S	V	G	E	G	N	V	A	A	E	T	D	N	E
ROMK1	M	T	S	A	F	L	F	S	L	E	T	Q	V	T	I	G	Y	G	F	R	V	T	E	Q	C	A	
IRK1	E	T	A	A	E	L	E	S	I	E	T	Q	T	T	I	G	Y	G	F	R	C	V	T	D	E	C	P
GIRK1	E	P	S	A	F	L	F	F	I	E	T	E	A	T	I	G	Y	G	Y	R	I	T	D	K	C	P	

FIG. 2A

TWIK-1	1	MLOS	LAGSS	CVREVE	-----	RHRS	AWCF	--GE	-----	LVLGY
f17c8	1	MYTDE	GEYS	SGD	TDHG	GS	TMOK	MSPN	TRON	FRONVNVVV
M110-2	1	MTVS	MEEN	SK	FM	LS	ATSK	DK	KVAT	DRSLLNKYHLGPIALHTGLVLS
TWIK-1	31	LEYL	YFG	AV	VFS	SVEL	PYED	LLROE	-----	LRKLKRRFLEEHEC---L
f17c8	47	LVEN	LIG	AG	LF	-----	-----	-----	-----	YLAETONSSES
M110-2	49	MTYA	LGG	AY	EFL	ST	EH	P	EEL	KRREKAIREFQDLKQOQMCNITSGIEN
TWIK-1	71	SEQO	EO	FL	GR	VL	-----	EASN	GV	SVLSNASGNWNW--DFTSALT
f17c8	69	LNEN	SEV	--	SKCL	HNL	PIG	GKI	TAEM	KS
M110-2	96	SEQS	EE	Y	TK	KE	TL	MLED	AH	NAHA
P1										
TWIK-1	110	FAS	TV	ST	GY	GHT	VP	ES	DG	GK
f17c8	115	F	W	T	L	Y	S	T	V	G
M110-2	142	F	T	T	T	V	I	P	V	G
TWIK-1	157	TVH	---	V	T	R	R	P	V	L
f17c8	162	AH	FL	V	V	S	N	R	T	R
M110-2	189	AQL	---	V	T	R	---	---	---	---
P2										
TWIK-1	197	FE	-	PA	AV	F	S	--	VL	-
f17c8	209	LL	C	S	E	S	L	S	S	A
M110-2	213	FAY	P	E	V	V	G	F	---	---
TWIK-1	239	QK	F	R	E	E	Y	K	I	G
f17c8	254	---	---	---	---	---	---	---	---	---
M110-2	253	---	---	---	---	---	---	---	---	---
TWIK-1	278	---	---	---	---	---	---	---	---	---
f17c8	295	LL	R	E	-	E	D	D	G	F
M110-2	294	KAK	E	L	A	G	K	M	F	O
TWIK-1	315	OK	N	E	P	F	V	A	T	---
f17c8	338	EK	L	S	S	L	E	S	T	---
M110-2	342	ED	G	L	I	A	F	S	P	D

FIG. 2B

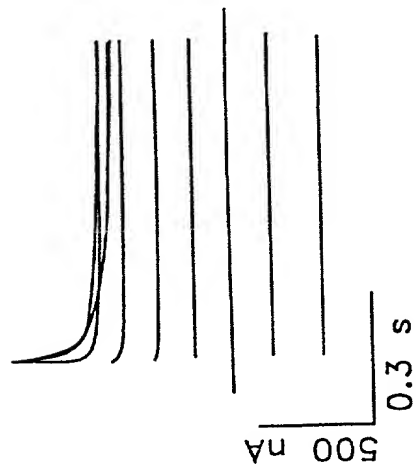


FIG. 3a

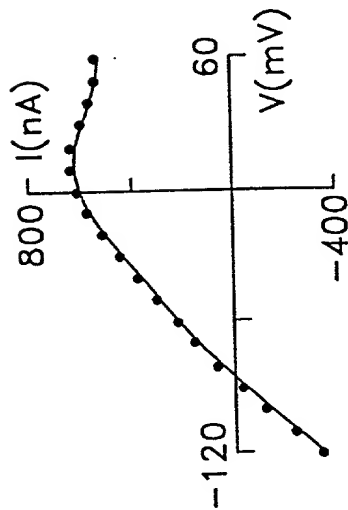


FIG. 3b

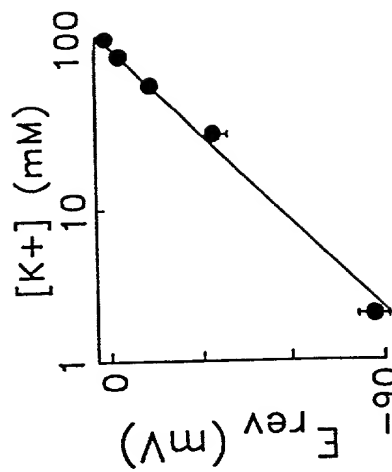


FIG. 3c

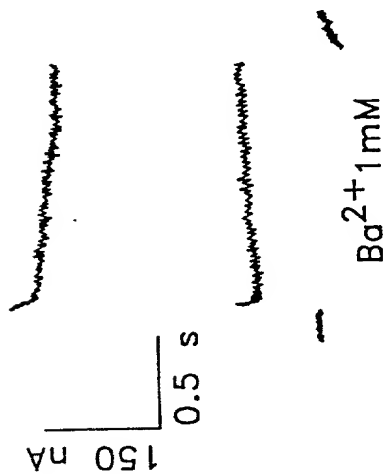


FIG. 3d

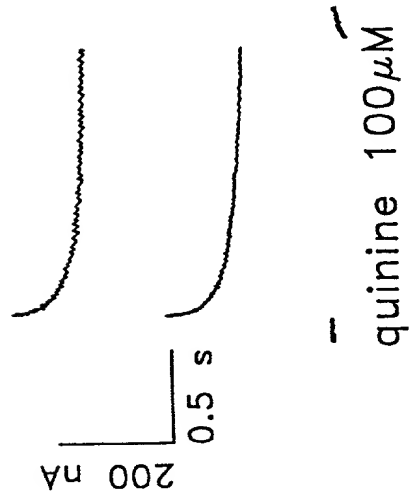


FIG. 3e

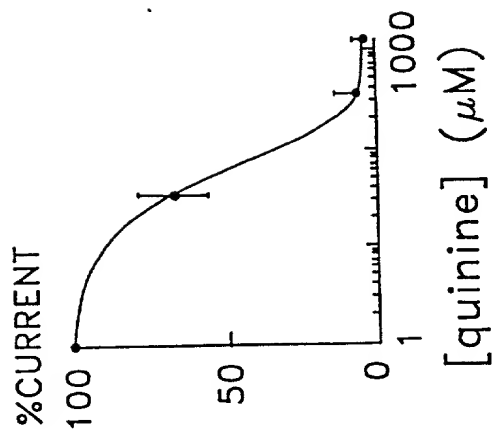
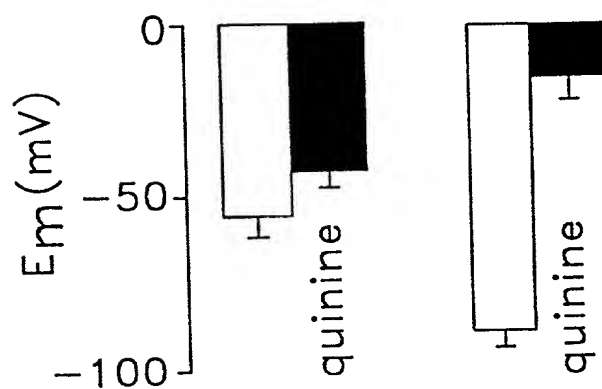


FIG. 3f



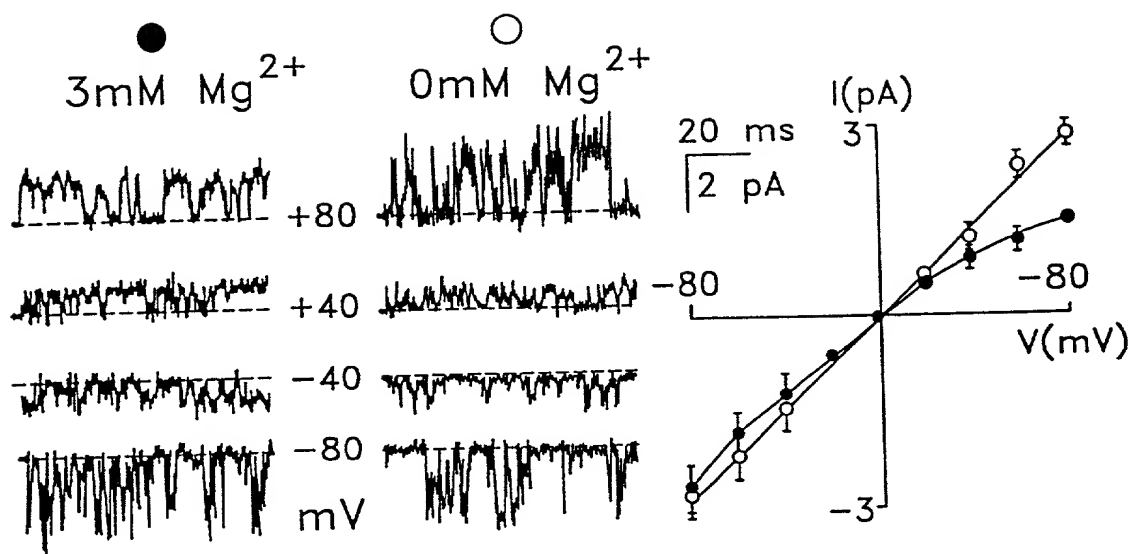


FIG. 5a

FIG. 5b

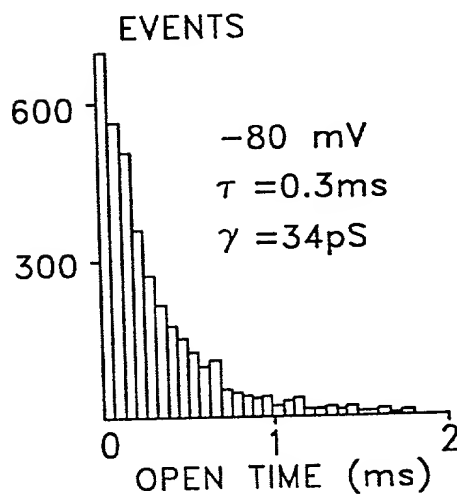
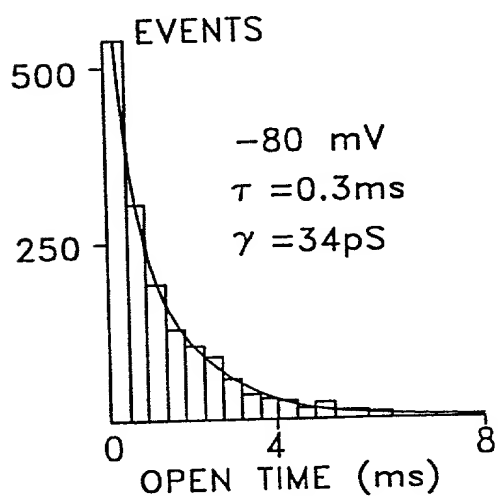
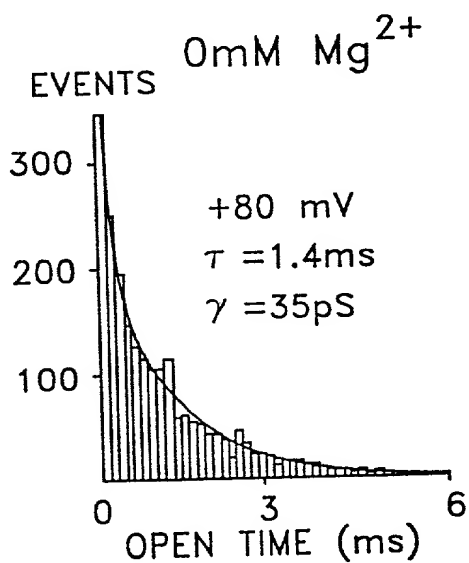
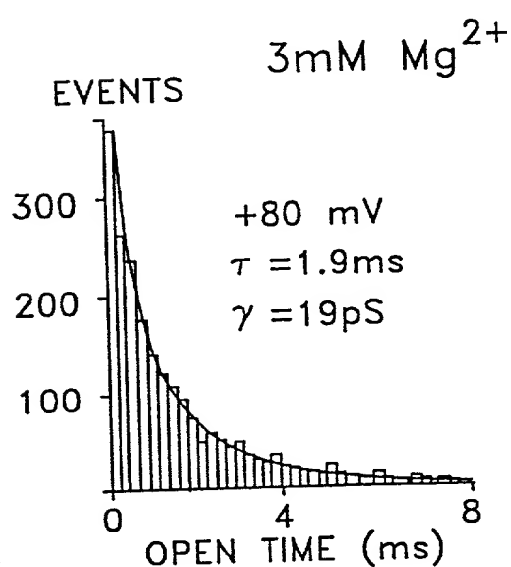


FIG. 5c

FIG. 5d

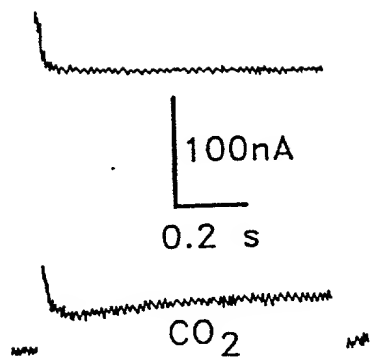


FIG. 6a

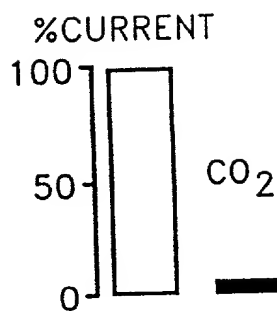


FIG. 6b

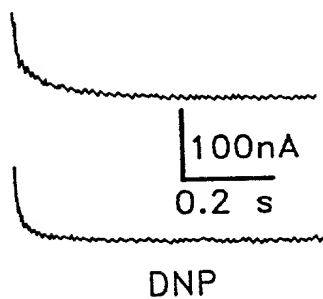


FIG. 6c

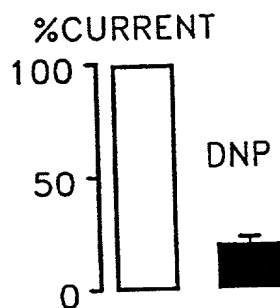


FIG. 6d

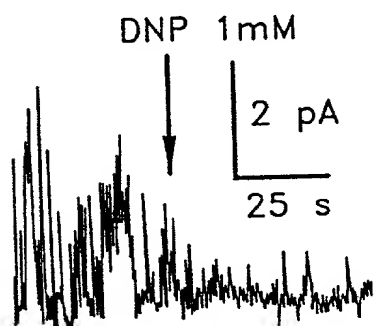


FIG. 6e

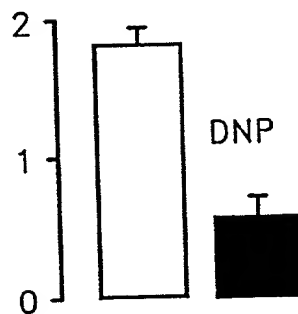


FIG. 6f

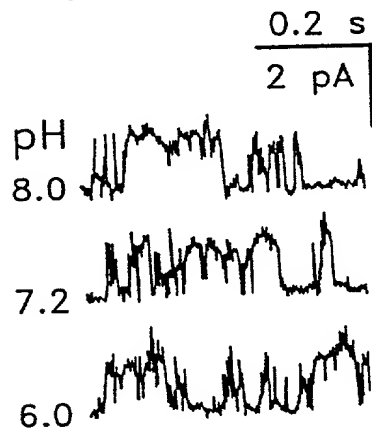


FIG. 6g

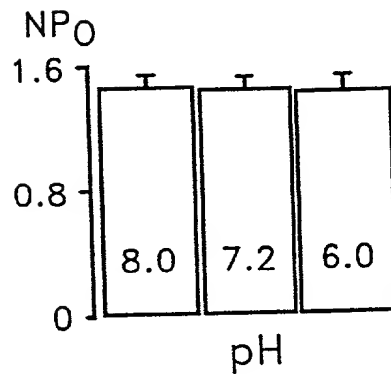


FIG. 6h

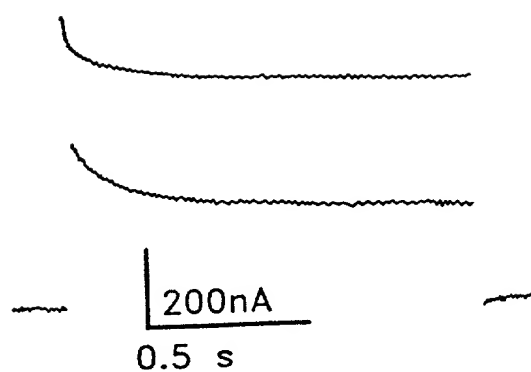


FIG. 7a

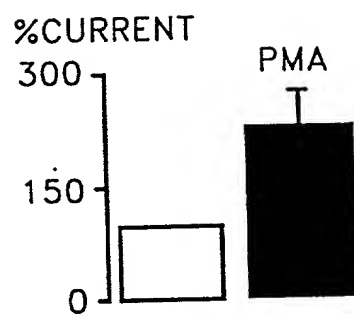


FIG. 7b

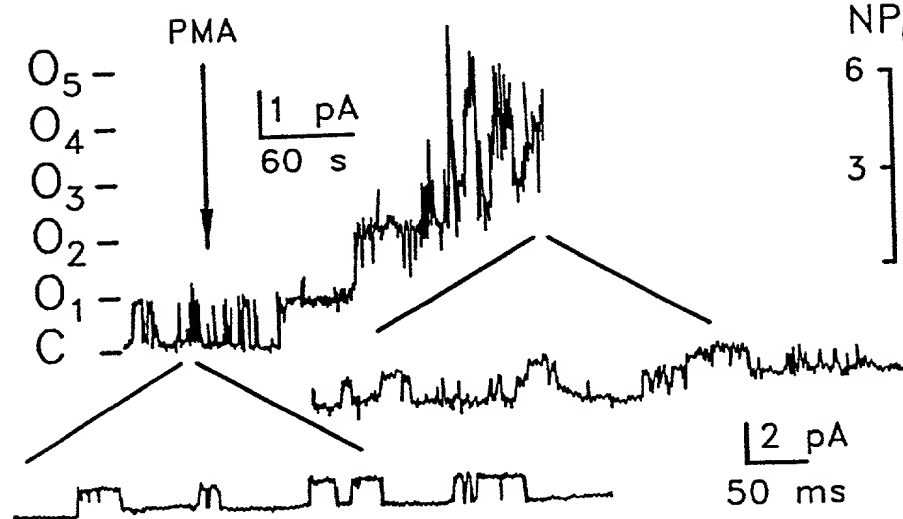


FIG. 7c

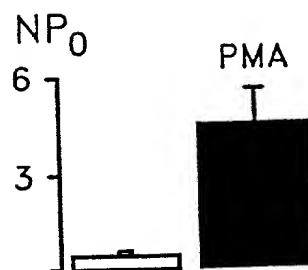


FIG. 7d

ASSOCIATE POWER OF ATTORNEY

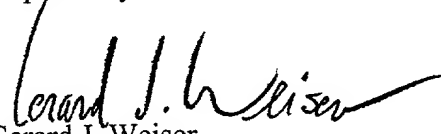
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

The undersigned hereby appoints Austin R. Miller, Reg. No. 16,602; James A. Droble Reg. No. 19,690; T. Daniel Christenbury, Reg. No. 31,750; Joan T. Kluger, Reg. No. 38,940; Patrick J. Farley, Reg. No. 42,524; Michael Patene, Ref. No. 42,982; David A. Sasso, Reg. No. 43,084; Kim R. Jessum, Reg. No. 43,694; Sharon Fenick, Reg. No. 45,269; Robert A. McKinley, Reg. No. 43,793, as associate attorneys to prosecute the captioned application and to transact business in the Patent and Trademark Office connected therewith. All further correspondence should be sent to:

Gerard J. Weiser
The Intellectual Property Department
Schnader Harrison Segal & Lewis, LLP
36th Floor
1600 Market Street
Philadelphia, Pa 19103
Telephone: (215) 563-1810

Respectfully submitted,


Gerard J. Weiser
Reg. No. 19,763

Date: 1/05/00

Attorney for Applicant

001110-00615450

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Lesage, Florian
Guillemare, Eric
Fink, Michel
Duprat, Fabrice
Lazdunki, Michel
Romey, Georges
Barhanin, Jacques
- (ii) TITLE OF INVENTION: FAMILY OF MAMMALIAN POTASSIUM CHANNELS,
THEIR CLONING AND THEIR USE ESPECIALLY FOR THE SCREENING
OF DRUGS
- (iii) NUMBER OF SEQUENCES: 19
- (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: WEISER & ASSOCIATES
(B) STREET: 230 South Fifteenth Street, Suite 500
(C) CITY: Philadelphia
(D) STATE: PA
(E) COUNTRY: USA
(F) ZIP: 19102
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/749,816
(B) FILING DATE: 15-NOV-1996
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Weiser, Gerard J.
(B) REGISTRATION NUMBER: 19,763
(C) REFERENCE/DOCKET NUMBER: 989.6351P
- (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 215-875-8383
(B) TELEFAX: 215-875-8394

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1894 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (ix) FEATURE:
(A) NAME/KEY: CDS

(B) LOCATION: 183..1190

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGGCAGGAAG	ACGGCGCTGC	CCGGAGGAGC	GGGGCGGGCG	GGCGCGCGGG	GGAGCGGGCG	60
CGGGGCGGGA	GCCAGGCCCG	GGCGGGGGCG	GGGGCGGGCG	GGCCAGAAGA	GGCGGCGGGC	120
CGCGCTCCGG	CCGGTCTGCG	GCGTTGGCCT	TGGCTTTGGC	TTTGGCGGCG	GCGGTGGAGA	180
AG ATG CTG CAG TCC CTG GCC GGC AGC TCG TGC GTG CGC CTG GTG GAG						227
Met Leu Gln Ser Leu Ala Gly Ser Ser Cys Val Arg Leu Val Glu						
1 5 10 15						
CGG CAC CGC TCG GCC TGG TGC TTC GGC TTC CTG GTG CTG GGC TAC TTG						275
Arg His Arg Ser Ala Trp Cys Phe Gly Phe Leu Val Leu Gly Tyr Leu						
20 25 30						
CTC TAC CTG GTC TTC GGC GCA GTG GTC TTC TCC TCG GTG GAG CTG CCC						323
Leu Tyr Leu Val Phe Gly Ala Val Val Phe Ser Ser Val Glu Leu Pro						
35 40 45						
TAT GAG GAC CTG CTG CGC CAG GAG CTG CGC AAG CTG AAG CGA CGC TTC						371
Tyr Glu Asp Leu Leu Arg Gln Glu Leu Arg Lys Leu Lys Arg Arg Phe						
50 55 60						
TTG GAG GAG CAC GAG TGC CTG TCT GAG CAG CAG CTG GAG CAG TTC CTG						419
Leu Glu Glu His Glu Cys Leu Ser Glu Gln Gln Leu Glu Gln Phe Leu						
65 70 75						
GGC CGG GTG CTG GAG GCC AGC AAC TAC GGC GTG TCG GTG CTC AGC AAC						467
Gly Arg Val Leu Glu Ala Ser Asn Tyr Gly Val Ser Val Leu Ser Asn						
80 85 90 95						
GCC TCG GGC AAC TGG AAC TGG GAC TTC ACC TCC GCG CTC TTC TTC GCC						515
Ala Ser Gly Asn Trp Asn Trp Asp Phe Thr Ser Ala Leu Phe Phe Ala						
100 105 110						
AGC ACC GTG CTC TCC ACC ACA GGT TAT GGC CAC ACC GTG CCC TTG TCA						563
Ser Thr Val Leu Ser Thr Thr Gly Tyr Gly His Thr Val Pro Leu Ser						
115 120 125						
GAT GGA GGT AAG GCC TTC TGC ATC ATC TAC TCC GTC ATT GGC ATT CCC						611
Asp Gly Gly Lys Ala Phe Cys Ile Ile Tyr Ser Val Ile Gly Ile Pro						
130 135 140						
TTC ACC CTC CTG TTC CTG ACG GCT GTG GTC CAG CGC ATC ACC GTG CAC						659
Phe Thr Leu Leu Phe Leu Thr Ala Val Val Gln Arg Ile Thr Val His						
145 150 155						
GTC ACC CGC AGG CCG GTC CTC TAC TTC CAC ATC CGC TGG GGC TTC TCC						707
Val Thr Arg Arg Pro Val Leu Tyr Phe His Ile Arg Trp Gly Phe Ser						
160 165 170 175						
AAG CAG GTG GTG GCC ATC GTC CAT GCC GTG CTC CTT GGG TTT GTC ACT						755
Lys Gln Val Val Ala Ile Val His Ala Val Leu Leu Gly Phe Val Thr						
180 185 190						

GTG TCC TGC TTC TTC TTC ATC CCG GCC GCT GTC TTC TCA GTC CTG GAG Val Ser Cys Phe Phe Phe Ile Pro Ala Ala Val Phe Ser Val Leu Glu 195 200 205	803
GAT GAC TGG AAC TTC CTG GAA TCC TTT TAT TTT TGT TTT ATT TCC CTG Asp Asp Trp Asn Phe Leu Glu Ser Phe Tyr Phe Cys Phe Ile Ser Leu 210 215 220	851
AGC ACC ATT GGC CTG GGG GAT TAT GTG CCT GGG GAA GGC TAC AAT CAA Ser Thr Ile Gly Leu Gly Asp Tyr Val Pro Gly Glu Gly Tyr Asn Gln 225 230 235	899
AAA TTC AGA GAG CTC TAT AAG ATT GGG ATC ACG TGT TAC CTG CTA CTT Lys Phe Arg Glu Leu Tyr Lys Ile Gly Ile Thr Cys Tyr Leu Leu Leu 240 245 250 255	947
GGC CTT ATT GCC ATG TTG GTA GTT CTG GAA ACC TTC TGT GAA CTC CAT Gly Leu Ile Ala Met Leu Val Val Leu Glu Thr Phe Cys Glu Leu His 260 265 270	995
GAG CTG AAA AAA TTC AGA AAA ATG TTC TAT GTG AAG AAG GAC AAG GAC Glu Leu Lys Lys Phe Arg Lys Met Phe Tyr Val Lys Lys Asp Lys Asp 275 280 285	1043
GAG GAT CAG GTG CAC ATC ATA GAG CAT GAC CAA CTG TCC TTC TCC TCG Glu Asp Gln Val His Ile Ile Glu His Asp Gln Leu Ser Phe Ser Ser 290 295 300	1091
ATC ACA GAC CAG GCA GCT GGC ATG AAA GAG GAC CAG AAG CAA AAT GAG Ile Thr Asp Gln Ala Ala Gly Met Lys Glu Asp Gln Lys Gln Asn Glu 305 310 315	1139
CCT TTT GTG GCC ACC CAG TCA TCT GCC TGC GTG GAT GGC CCT GCA AAC Pro Phe Val Ala Thr Gln Ser Ser Ala Cys Val Asp Gly Pro Ala Asn 320 325 330 335	1187
CAT TGAGCGTAGG ATTTGTTGCA TTATGCTAGA GCACCAGGGT CAGGGTGCAA His	1240
GGAAGAGGCT TAAGTATGTT CATTTTTATC AGAATGCAAA AGCGAAAATT ATGTCACTTT	1300
AAGAAATAGC TACTGTTTGC AATGTCTTAT TAAAAACAA CAAAAAAGA CACATGGAAC	1360
AAAGAAGCTG TGACCCAGC AGGATGTCTA ATATGTGAGG AAATGAGATG TCCACCTAAA	1420
ATTCATATGT GACAAAATTA TCTCGACCTT ACATAGGAGG AGAATACTTG AAGCAGTATG	1480
CTGCTGTGGT TAGAAGCAGA TTTTATACTT TTAAGTGGAA ACTTTGGGGT TTGCATTTAG	1540
ATCATTTAGC TGATGGCTAA ATAGCAAAAT TTATATTTAG AAGCAAAAAA AAAAAGCATA	1600
GAGATGTGTT TTATAAATAG GTTTATGTGT ACTGGTTTGC ATGTACCCAC CCAAATGAT	1660
TATTTTTGGA GAATCTAAGT CAAACTCACT ATTTATAATG CATAGGTAAC CATTAACTAT	1720
GTACATATAA AGTATAAATA TGTTTATATT CTGTACATAT GGTTTAGGTC ACCAGATCCT	1780
AGTGTAGTTC TGAAACTAAG ACTATAGATA TTTTGTCTTCT TTTGATTCTT CTTTATACTA	1840
AAGAATCCAG AGTTGCTACA ATAAAATAAG GGGAATAATA AAAAAAAAAA AAAA	1894

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 336 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Leu Gln Ser Leu Ala Gly Ser Ser Cys Val Arg Leu Val Glu Arg
1 5 10 15
His Arg Ser Ala Trp Cys Phe Gly Phe Leu Val Leu Gly Tyr Leu Leu
20 25 30
Tyr Leu Val Phe Gly Ala Val Val Phe Ser Ser Val Glu Leu Pro Tyr
35 40 45
Glu Asp Leu Leu Arg Gln Glu Leu Arg Lys Leu Lys Arg Arg Phe Leu
50 55 60
Glu Glu His Glu Cys Leu Ser Glu Gln Gln Leu Glu Gln Phe Leu Gly
65 70 75 80
Arg Val Leu Glu Ala Ser Asn Tyr Gly Val Ser Val Leu Ser Asn Ala
85 90 95
Ser Gly Asn Trp Asn Trp Asp Phe Thr Ser Ala Leu Phe Phe Ala Ser
100 105 110
Thr Val Leu Ser Thr Thr Gly Tyr Gly His Thr Val Pro Leu Ser Asp
115 120 125
Gly Gly Lys Ala Phe Cys Ile Ile Tyr Ser Val Ile Gly Ile Pro Phe
130 135 140
Thr Leu Leu Phe Leu Thr Ala Val Val Gln Arg Ile Thr Val His Val
145 150 155 160
Thr Arg Arg Pro Val Leu Tyr Phe His Ile Arg Trp Gly Phe Ser Lys
165 170 175
Gln Val Val Ala Ile Val His Ala Val Leu Leu Gly Phe Val Thr Val
180 185 190
Ser Cys Phe Phe Phe Ile Pro Ala Ala Val Phe Ser Val Leu Glu Asp
195 200 205
Asp Trp Asn Phe Leu Glu Ser Phe Tyr Phe Cys Phe Ile Ser Leu Ser
210 215 220
Thr Ile Gly Leu Gly Asp Tyr Val Pro Gly Glu Gly Tyr Asn Gln Lys
225 230 235 240
Phe Arg Glu Leu Tyr Lys Ile Gly Ile Thr Cys Tyr Leu Leu Leu Gly
245 250 255

1. 100% (100%)
 2. 100% (100%)
 3. 100% (100%)
 4. 100% (100%)
 5. 100% (100%)
 6. 100% (100%)
 7. 100% (100%)
 8. 100% (100%)
 9. 100% (100%)
 10. 100% (100%)
 11. 100% (100%)
 12. 100% (100%)
 13. 100% (100%)
 14. 100% (100%)
 15. 100% (100%)
 16. 100% (100%)
 17. 100% (100%)
 18. 100% (100%)
 19. 100% (100%)
 20. 100% (100%)
 21. 100% (100%)
 22. 100% (100%)
 23. 100% (100%)
 24. 100% (100%)
 25. 100% (100%)
 26. 100% (100%)
 27. 100% (100%)
 28. 100% (100%)
 29. 100% (100%)
 30. 100% (100%)
 31. 100% (100%)
 32. 100% (100%)
 33. 100% (100%)
 34. 100% (100%)
 35. 100% (100%)
 36. 100% (100%)
 37. 100% (100%)
 38. 100% (100%)
 39. 100% (100%)
 40. 100% (100%)
 41. 100% (100%)
 42. 100% (100%)
 43. 100% (100%)
 44. 100% (100%)
 45. 100% (100%)
 46. 100% (100%)
 47. 100% (100%)
 48. 100% (100%)
 49. 100% (100%)
 50. 100% (100%)
 51. 100% (100%)
 52. 100% (100%)
 53. 100% (100%)
 54. 100% (100%)
 55. 100% (100%)
 56. 100% (100%)
 57. 100% (100%)
 58. 100% (100%)
 59. 100% (100%)
 60. 100% (100%)
 61. 100% (100%)
 62. 100% (100%)
 63. 100% (100%)
 64. 100% (100%)
 65. 100% (100%)
 66. 100% (100%)
 67. 100% (100%)
 68. 100% (100%)
 69. 100% (100%)
 70. 100% (100%)
 71. 100% (100%)
 72. 100% (100%)
 73. 100% (100%)
 74. 100% (100%)
 75. 100% (100%)
 76. 100% (100%)
 77. 100% (100%)
 78. 100% (100%)
 79. 100% (100%)
 80. 100% (100%)
 81. 100% (100%)
 82. 100% (100%)
 83. 100% (100%)
 84. 100% (100%)
 85. 100% (100%)
 86. 100% (100%)
 87. 100% (100%)
 88. 100% (100%)
 89. 100% (100%)
 90. 100% (100%)
 91. 100% (100%)
 92. 100% (100%)
 93. 100% (100%)
 94. 100% (100%)
 95. 100% (100%)
 96. 100% (100%)
 97. 100% (100%)
 98. 100% (100%)
 99. 100% (100%)
 100. 100% (100%)

(i) SEQUENCE CHARACTERISTICS:

- (ii) MOLECULE TYPE: protein

Met 1	Tyr	Thr	Asp	Glu 5	Gly	Glu	Tyr	Ser	Gly 10	Asp	Thr	Asp	His	Gly 15	Gly
Ser	Thr	Met	Gln 20	Lys	Met	Ser	Pro	Asn 25	Thr	Arg	Gln	Asn	Phe 30	Arg	Gln
Asn	Val	Asn 35	Val	Val	Val	Cys	Leu 40	Ser	Ala	Ala	Ile	Thr 45	Leu	Leu	Val
Phe 50	Asn	Leu	Ile	Gly	Ala	Gly 55	Ile	Phe	Tyr	Leu	Ala 60	Glu	Thr	Gln	Asn
Ser 65	Ser	Glu	Ser	Leu	Asn 70	Glu	Asn	Ser	Glu	Val 75	Ser	Lys	Cys	Leu	His 80
Asn	Leu	Pro	Ile	Gly 85	Gly	Lys	Ile	Thr	Ala 90	Glu	Met	Lys	Ser	Lys 95	Leu
Gly	Lys	Cys	Leu 100	Thr	Lys	Ser	Ser	Arg 105	Ile	Asp	Gly	Phe	Gly 110	Lys	Ala
Ile	Phe 115	Phe	Ser	Trp	Thr	Leu	Tyr 120	Ser	Thr	Val	Gly 125	Tyr	Gly	Ser	Leu
Tyr	Pro 130	His	Ser	Thr	Leu	Gly 135	Arg	Tyr	Leu	Thr	Ile 140	Phe	Tyr	Ser	Leu
Leu 145	Met	Ile	Pro	Val	Phe 150	Ile	Ala	Phe	Lys	Phe 155	Glu	Phe	Gly	Thr	Phe 160
Leu	Ala	His	Phe	Leu 165	Val	Val	Val	Ser	Asn 170	Arg	Thr	Arg	Leu	Ala 175	Val

Lys Lys Ala Tyr Tyr Lys Leu Ser Gln Asn Pro Glu Asn Ala Glu Thr
 180 185 190
 Pro Ser Asn Ser Leu Gln His Asp Tyr Leu Ile Phe Leu Ser Ser Leu
 195 200 205
 Leu Leu Cys Ser Ile Ser Leu Leu Ser Ser Ser Ala Leu Phe Ser Ser
 210 215 220
 Ile Glu Asn Ile Ser Tyr Leu Ser Ser Val Tyr Phe Gly Ile Ile Thr
 225 230 235 240
 Met Phe Leu Ile Gly Ile Gly Asp Ile Val Pro Thr Asn Leu Val Trp
 245 250 255
 Phe Ser Gly Tyr Cys Met Leu Phe Leu Ile Ser Asp Val Leu Ser Asn
 260 265 270
 Gln Ile Phe Tyr Phe Cys Gln Ala Arg Val Arg Tyr Phe Phe His Ile
 275 280 285
 Leu Ala Arg Lys Ile Leu Leu Leu Arg Glu Glu Asp Asp Gly Phe Gln
 290 295 300
 Leu Glu Thr Thr Val Ser Leu Gln His Ile Pro Ile Ile Asn Ser Gln
 305 310 315 320
 Cys Met Pro Ser Leu Val Leu Asp Cys Glu Lys Glu Glu Leu Asp Asn
 325 330 335
 Asp Glu Lys Leu Ile Ser Ser Leu Thr Ser Thr
 340 345

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 383 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Thr Val Ser Met Glu Glu Asn Ser Lys Ile Gln Met Leu Ser Ala
 1 5 10 15
 Thr Ser Lys Asp Lys Lys Val Ala Thr Asp Arg Ser Leu Leu Asn Lys
 20 25 30
 Tyr His Leu Gly Pro Leu Ala Leu His Thr Gly Leu Val Leu Ser Cys
 35 40 45
 Val Thr Tyr Ala Leu Gly Gly Ala Tyr Leu Phe Leu Ser Ile Glu His
 50 55 60

Pro	Glu	Glu	Leu	Lys	Arg	Arg	Glu	Lys	Ala	Ile	Arg	Glu	Phe	Gln	Asp	65	70	75	80
Leu	Lys	Gln	Gln	Phe	Met	Gly	Asn	Ile	Thr	Ser	Gly	Ile	Glu	Asn	Ser	85	90	95	
Glu	Gln	Ser	Ile	Glu	Ile	Tyr	Thr	Lys	Lys	Leu	Ile	Leu	Met	Leu	Glu	100	105	110	
Asp	Ala	His	Asn	Ala	His	Ala	Phe	Glu	Tyr	Phe	Phe	Leu	Asn	His	Glu	115	120	125	
Ile	Pro	Lys	Asp	Met	Trp	Thr	Phe	Ser	Ser	Ala	Leu	Val	Phe	Thr	Thr	130	135	140	
Thr	Thr	Val	Ile	Pro	Val	Gly	Tyr	Gly	Tyr	Ile	Phe	Pro	Val	Ser	Ala	145	150	155	160
Tyr	Gly	Arg	Met	Cys	Leu	Ile	Ala	Tyr	Ala	Leu	Leu	Gly	Ile	Pro	Leu	165	170	175	
Thr	Leu	Val	Thr	Met	Ala	Asp	Thr	Gly	Lys	Phe	Ala	Ala	Gln	Leu	Val	180	185	190	
Thr	Arg	Trp	Phe	Gly	Asp	Asn	Asn	Met	Ala	Ile	Pro	Ala	Ala	Ile	Phe	195	200	205	
Val	Cys	Leu	Leu	Phe	Ala	Tyr	Pro	Leu	Val	Val	Gly	Phe	Ile	Leu	Cys	210	215	220	
Ser	Thr	Ser	Asn	Ile	Thr	Tyr	Leu	Asp	Ser	Val	Tyr	Phe	Ser	Leu	Thr	225	230	235	240
Ser	Ile	Phe	Thr	Ile	Gly	Phe	Gly	Asp	Leu	Thr	Pro	Asp	Met	Asn	Val	245	250	255	
Ile	His	Met	Val	Leu	Phe	Leu	Ala	Val	Gly	Val	Ile	Leu	Val	Thr	Ile	260	265	270	
Thr	Leu	Asp	Ile	Val	Ala	Ala	Glu	Met	Ile	Asp	Arg	Val	His	Tyr	Met	275	280	285	
Gly	Arg	His	Val	Gly	Lys	Ala	Lys	Glu	Leu	Ala	Gly	Lys	Met	Phe	Gln	290	295	300	
Leu	Ala	Gln	Ser	Leu	Asn	Met	Lys	Gln	Gly	Leu	Val	Ser	Gly	Val	Gly	305	310	315	320
Gln	Leu	His	Ala	Leu	Ala	Arg	Phe	Gly	Met	Leu	Val	Gly	Arg	Glu	Glu	325	330	335	
Val	Asp	Lys	Thr	Gln	Glu	Asp	Gly	Ile	Ile	Ala	Phe	Ser	Pro	Asp	Val	340	345	350	
Met	Asp	Gly	Leu	Glu	Phe	Met	Asp	Thr	Leu	Ser	Ile	Tyr	Ser	Arg	Arg	355	360	365	
Ser	Arg	Arg	Ser	Ala	Glu	Asn	Ser	Ala	Arg	Asn	Leu	Phe	Leu	Ser		370	375	380	

(2) INFORMATION FOR SEQ ID NO:5:

[illegible]

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

(2) INFORMATION FOR SEQ ID NO:6:

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

(2) INFORMATION FOR SEQ ID NO:7:

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

27

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Tyr Gly Asn Ala Leu Tyr Phe Cys Thr Val Ser Leu Leu Thr Val Gly
1 5 10 15
Leu Gly Asp Ile Leu Pro Lys Ser Val Gly Ala
 20 25

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Tyr Trp Thr Cys Val Tyr Phe Leu Ile Val Thr Met Ser Thr Val Gly
1 5 10 15
Tyr Gly Asp Val Tyr Cys Glu Thr Val Leu Gly
 20 25

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ile Pro Asp Ala Phe Trp Trp Ala Val Val Thr Met Thr Thr Val Gly
1 5 10 15

Variable	Mean	Standard Deviation	Minimum	Maximum
Age	34.5	10.2	22	55
Gender	0.5	0.5	0	1
Marital Status	0.6	0.5	0	1
Education	12.5	1.5	10	15
Income	3500	1500	1000	7000
Health	0.8	0.2	0	1
Smoking	0.3	0.5	0	1
Alcohol	0.2	0.4	0	1
Exercise	0.4	0.5	0	1
Stress	0.6	0.5	0	1
Sleep	0.7	0.3	0	1
Diet	0.5	0.5	0	1
Work	0.8	0.2	0	1
Family	0.6	0.5	0	1
Friends	0.7	0.4	0	1
Hobbies	0.5	0.5	0	1
Travel	0.4	0.5	0	1
Religion	0.6	0.5	0	1
Politics	0.5	0.5	0	1
Environment	0.7	0.4	0	1
Technology	0.8	0.3	0	1
Art	0.6	0.5	0	1
Music	0.7	0.4	0	1
Sports	0.5	0.5	0	1
Gardening	0.4	0.5	0	1
Reading	0.6	0.5	0	1
Writing	0.3	0.5	0	1
Volunteering	0.2	0.4	0	1
Charitable	0.1	0.3	0	1
Philanthropy	0.05	0.2	0	1
Activism	0.15	0.35	0	1
Leadership	0.25	0.45	0	1
Entrepreneurship	0.1	0.3	0	1
Investment	0.3	0.5	0	1
Real Estate	0.2	0.4	0	1
Stocks	0.15	0.35	0	1
Bonds	0.1	0.3	0	1
Commodities	0.05	0.2	0	1
Cryptocurrency	0.02	0.15	0	1
Art Collection	0.01	0.1	0	1
Real Estate Collection	0.005	0.05	0	1
Stock Collection	0.002	0.02	0	1
Bond Collection	0.001	0.01	0	1
Commodity Collection	0.0005	0.005	0	1
Cryptocurrency Collection	0.0001	0.001	0	1
Art Collection Value	100000	50000	0	500000
Real Estate Collection Value	200000	100000	0	1000000
Stock Collection Value	50000	25000	0	250000
Bond Collection Value	25000	12500	0	125000
Commodity Collection Value	12500	6250	0	62500
Cryptocurrency Collection Value	6250	3125	0	31250
Art Collection Return	0.05	0.02	0	0.1
Real Estate Collection Return	0.02	0.01	0	0.05
Stock Collection Return	0.01	0.005	0	0.03
Bond Collection Return	0.005	0.002	0	0.015
Commodity Collection Return	0.002	0.001	0	0.006
Cryptocurrency Collection Return	0.001	0.0005	0	0.003

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Tyr Gly Asp Ile Cys Pro Thr Thr Ala Leu Gly
20 25

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Tyr Gly Asp Met Val Pro Glu Thr Ile Ala Gly
20 25

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

29

Tyr Gly Asp Met Ala Pro Lys Thr Tyr Ile Gly
20 25

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Tyr Val Thr Ala Leu Tyr Trp Ser Ile Thr Thr Leu Thr Thr Thr Gly
1 5 10 15

Tyr Gly Asp Phe His Ala Glu Asn Pro Arg Glu
20 25

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Tyr Val Thr Ser Met Tyr Trp Ser Ile Thr Thr Leu Thr Thr Val Gly
1 5 10 15

Tyr Gly Asp Leu His Pro Val Asn Thr Lys Glu
20 25

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Phe Gly Asn Val Ala Ala Glu Thr Asp Asn Glu
20 25

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Thr Ser Ala Phe Leu Phe Ser Leu Glu Thr Gln Val Thr Ile Gly
1 5 10 15

Tyr Gly Phe Arg Phe Val Thr Glu Gln Cys Ala
20 25

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Phe Thr Ala Ala Phe Leu Phe Ser Ile Glu Thr Gln Thr Thr Ile Gly
1 5 10 15

Tyr Gly Phe Arg Cys Val Thr Asp Glu Cys Pro
20 25

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

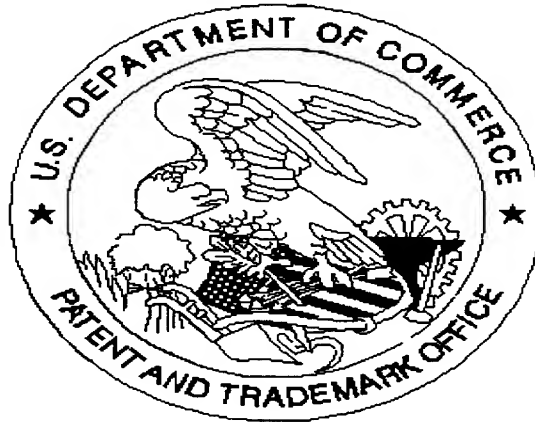
- (A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

Variable	Mean	SD	Min	Max
Age	34.5	10.2	21	55
Gender	0.5	0.5	0	1
Marital status	0.6	0.5	0	1
Education	12.5	1.5	9	16
Income	1500	500	500	3000
Health status	0.7	0.5	0	1
Smoking status	0.3	0.5	0	1
Alcohol consumption	0.2	0.4	0	1
Exercise frequency	0.4	0.5	0	1
Stress level	0.6	0.5	0	1
Sleep quality	0.5	0.5	0	1
Work satisfaction	0.4	0.5	0	1
Life satisfaction	0.5	0.5	0	1
Depression score	0.3	0.4	0	1
Anxiety score	0.2	0.3	0	1
Resilience score	0.6	0.5	0	1
Optimism score	0.5	0.5	0	1
Gratitude score	0.4	0.5	0	1
Forgiveness score	0.3	0.4	0	1
Self-compassion score	0.4	0.5	0	1
Emotional regulation score	0.5	0.5	0	1
Prosocial behavior score	0.4	0.5	0	1
Life purpose score	0.3	0.4	0	1
Meaning in life score	0.4	0.5	0	1
Existential well-being score	0.3	0.4	0	1
Overall well-being score	0.4	0.5	0	1

Tyr Gly Tyr Arg Tyr Ile Thr Asp Lys Cys Pro
20 25

United States Patent & Trademark Office
Office of Initial Patent Examination -- Scanning Division



Application deficiencies were found during scanning:

☐ Page(s) _____ of Declaration were not present
for scanning. (Document title)

☐ Page(s) _____ of _____ were not present
for scanning. (Document title)

☐ Scanned copy is best available.